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THE REACTIONS BETWEEN BACTERIA AND ANIMAL TISSUES UNDER CONDITIONS OF ARTIFICIAL CULTIVATION.

II. Bactericidal Action in Tissue Cultures.

By HENRY FIELD SMYTH, M.D.

(*From the Laboratory of Hygiene of the University of Pennsylvania, Philadelphia.*)

(Received for publication, October 14, 1915.)

The author has previously[1] reported investigations on the reactions observed when tissue cultures *in vitro* were inoculated with living pathogenic bacteria, and he desires here to record further observations on the subject. In the former experiments the culture medium used was chicken plasma two parts, with Ringer solution one part, with various chick embryo tissues. In the present experiments a dilution of equal parts of plasma and Ringer solution was employed, thus reducing the proportion of plasma and thus of antibodies or other substances inhibiting bacterial growth. With this change in the strength of the plasma corresponding changes were observed in the bacterial reactions, and the object of this paper is to report these changes, especially as observed with *Bacillus typhosus* and *Bacterium diphtheriticum*.

Bacillus typhosus.

In the former series the organism used was an old laboratory strain of the Wyatt-Johnson type, while in the present series in addition to this strain there were also employed three recently isolated strains from clinical typhoid cases.[2] With the stronger plasma first employed the Wyatt-Johnson bacillus refused to grow or even to survive for any length of time. In the present series with more dilute

[1] Smyth, H. F., *Jour. Exper. Med.*, 1915, xxi, 103; *Centralbl. f. Bacteriol., 1te Abt., Orig.*, 1915, lxxvi, 12.

[2] These strains were furnished by Dr. Ira Ayer, Pathologist of the Episcopal Hospital, Philadelphia.

plasma there were many purposely very heavy inoculations and in a number of instances some of the organisms survived and formed colonies. In the first series inoculations from 24 hour agar slants were made into a few cc. of Ringer solution, and 1, 2, or 3 loops from this were inoculated into the diluted plasma or into the amount of fresh Ringer solution to be used for dilution, while in the second series many inoculations were made directly from agar into diluted plasma. In all instances, however, there was evident great reduction in the number of organisms developing, and several tests were made to ascertain the extent of this bacteriolysis by the use of agar plates containing a similar amount of the same inoculated Ringer solution (0.05 to 0.1 cc.) to that used in the cultures. Table I, No. 42, and Table II, No. 44, show some of these results. No. 42 shows a great reduction, uncounted in the first dilution cultures and over 1,300 in the second dilution, no bacteria surviving in either. No. 44 also shows the same results, though here the inoculations were not so heavy. Series of cultures were also made with the other recently isolated strains, designated as A1, A2, and A3, and here also the same marked bacteriolytic action of the plasma was evident though not in as high a degree, some colonies always developing except in the second dilutions. The results with A1 are shown in Table II, No. 45. In the first dilution the colonies on the agar plates were too numerous to count, while the plasma cultures averaged little over 150 in the presence of tissue and showed only 27 colonies without tissue. In the second dilution where there was an average of over 90 colonies on agar plates none developed in the plasma cultures with heart tissue or without tissue, and an average of 5.5 colonies developed in the presence of splenic tissue. In Table I, No. 43, are shown similar results with Type A3. Here the first dilution contained 150,000 colonies on agar, while in plasma alone there were no colonies, in the presence of heart tissue an average of 1.5 colonies, and in the presence of splenic tissue 35 colonies per culture. The second dilution showed an average of 55 colonies on agar while no colonies developed in plasma with or without tissue.

The tables also show similar quantitative tests made for comparison with *Bacillus coli verus*, *Bacillus dysenteriæ* (Harris), and *Bacterium diphtheriticum*.

TABLE I.

Series 7. November 5, 1914. 24 Hour Plasma. 15 Day Chick Embryo Tissue.

Stock inoculations. 1 loop from 24 hour agar slant in 1 cc. of Ringer solution.
Dilution A. 1 " " stock inoculation " 1 " " " "
" B. 1 " " Dilution A " 1 " " " "

40s	*Bacterium diphtheriticum* inoculations.	
A	Heart tissue.	No bacterial colonies.
	" "	" " "
	Spleen "	Scattered colonies with halo around tissue.
	Plasma alone.	No colonies.
	Agar plates.	" "
B	Heart tissue.	" "
	" "	" "
	Spleen "	5 colonies just beyond area of tissue growth.
	Plasma alone.	No colonies.
	Agar plates.	" "
41s	*Bacillus coli verus* inoculations.	
A	Heart tissue.	Very numerous scattered colonies.
	" "	" " " "
	Spleen "	" " " " } no halos.
	Plasma alone.	" " " " }
	Agar plates.	Average over 9,000 colonies.
B	Heart tissue.	3 colonies.
	Spleen "	9 "
	Plasma alone.	1 colony.
	Agar plates.	17 colonies.
42s	*Bacillus typhosus* inoculations, Wyatt-Johnson strain.	
A	Heart tissue.	No colonies.
	" "	" "
	Spleen "	" "
	Plasma alone.	" "
	Agar plates.	Very numerous colonies, not counted.
B	Heart tissue.	No colonies.
	" "	" "
	Spleen "	" "
	Plasma alone.	" "
	Agar plates.	1,362 "
43s	*Bacillus typhosus* inoculations, A3 type.	
A	Heart tissue,	1 colony.
	" "	3 colonies.
	Spleen "	35 " around border of culture.
	Plasma alone.	No colonies.
	Agar plates.	150,000 colonies.
B	Heart tissue.	No colonies.
	" "	" "
	Spleen "	" "
	Plasma alone.	" "
	Agar plates.	55 "

TABLE II.

Series 8. November 9, 1914. 5 Day Plasma. 11 Day Chick Embryo Tissue.

Stock inoculations. 1 loop from 24 hour agar slant in 1 cc. of Ringer solution.
Dilution A. 2 loops " stock inoculation " 1 " " " "
" B. 2 " " Dilution A " 1 " " " "

44s	*Bacillus typhosus* inoculations, Wyatt-Johnson strain.	
A	Heart tissue.	No colonies.
	" "	" "
	Plasma alone.	" "
	Agar plates.	Very numerous colonies, not counted.
B	Heart tissue.	No colonies.
	" "	" "
	Spleen "	" "
	" "	" "
	Plasma alone.	" "
	Agar plates.	Average 68.5 colonies.
45s	*Bacillus typhosus* inoculations, A1 type.	
A	Heart tissue.	230 colonies, scattered.
	" "	82 " "
	Plasma alone.	27 "
	Agar plates.	Very numerous colonies, not counted.
B	Heart tissue.	No colonies.
	" "	" "
	Spleen "	3 "
	" "	8 " , no halo.
	Plasma alone.	No "
	Agar plates.	Average 92.5 colonies.
46s	*Bacillus dysenteriæ* (Harris) inoculations.	
A	Heart tissue.	137 colonies, scattered.
	" "	1,000+ " "
	Plasma alone.	200+ "
	Agar plates.	None made.
B	Heart tissue.	1 colony.
	" "	2 colonies.
	Spleen "	1 colony away from tissue.
	" "	6 colonies.
	Plasma alone.	No "
	Agar plates.	Average 157 colonies.

In this series the splenic tissue was too young to have much deleterious action on the organisms, as at 11 days there is very little free cell migration.

Bacillus coli verus.

Bacillus coli verus (Table I, No. 41) shows only a slight reduction; Dilution A was not counted, but there appeared to be as many colonies in plasma as in agar, and Dilution B shows an apparent reduction from an average of 17 colonies on agar to 9 with splenic tissue, 3 with heart tissue, and 1 with plasma alone, all of which might well be due to irregularities in distribution, as such small amounts of inoculated Ringer solution are used in making cultures. These results agree with those previously reported for this organism, though no actual counts were then made.

Bacillus dysenteriæ.

No tests were made with this organism in the former series. In the present series (Table II, No. 46) *Bacillus dysenteriæ* seems to occupy a position between the extremes of *Bacillus typhosus* and *Bacillus coli verus*, there being an evident marked bacteriolysis but decidedly less than with *Bacillus typhosus*. The average of 157 bacilli in Dilution B on agar was reduced in plasma with heart tissue to 1.5 and with splenic tissue to 3.5, while there were no colonies in plasma without tissue. There was also an evident reduction in Dilution A cultures, though no counts were made on agar.

Bacterium diphtheriticum.

In the former series with the stronger plasma three strains of *Bacterium diphtheriticum* were employed, Park-Williams Bacillus 8 and two freshly isolated strains from clinical cases. In the present series with the 50 per cent plasma ten different strains were used, Bacillus 8 and nine recently isolated organisms. In general the results agree with those already reported, though here also the effect of plasma dilution is seen in the survival of many organisms away from the presence of tissue, but even when many such do survive, the colonies are more numerous and larger around the tissue fragments. In the former series with 66 per cent plasma diphtheria colonies developed only closely surrounding tissue fragments, giving to each fragment the appearance of a sun in a miniature solar system. Table I, No. 40, and Tables III, IV, and V show the results of these cul-

tures. In Table IV larger box cultures, with two or more times as much plasma as in the cover-glass cultures designated as s, were used, and in Table V still larger cultures in Petri dishes, with 1 cc. of plasma. This accounts for the much higher bacterial counts in agar plates and lessens the influence of tissue cells as the amount of

TABLE III.

Series 13. January 15, 1915. 1 Day Plasma. 13 Day Chick Embryo Tissue.

Bacterium diphtheriticum inoculations. All cultures from 1st dilution.

82s	Strain 2 (Bacillus 8)	Heart tissue. " " Spleen " " "	5 colonies. 1 colony. 1 " not near tissue. 2 colonies " " "
83s	Strain 3	Heart " Spleen "	2 " near tissue. 1 colony not near tissue.
84s	" 4	Heart " Spleen " " "	2 colonies. 1 colony, not near tissue. 1 " " " "
85s	" 5	Heart " " " Spleen " " "	12 colonies, scattered. 23 " " 16 " not near tissue. 57 " " " "
86s	" 6	Heart " " " Spleen "	4 " , scattered. 2 " 2 " not near tissue.
87s	" 7	Heart " " " Spleen " " "	5 " , scattered. 3 " " 4 " " 9 " "
88s	" 8	Heart " Spleen "	3 " " 3 " "
89s	" 9	Heart tissue. " " Spleen " " "	Many scattered colonies. " " " " " " avoiding tissue. " " " " "
90s	" 10	Heart tissue.	2 colonies.

TABLE IV.

Series 14. January 20, 1915. Fresh Plasma. 14 Day Chick Embryo Tissue.

Bacterium diphtheriticum inoculations. All cultures from 1st dilution.

91B	Strain 2 (Bacillus 8)	Heart tissue.	No colonies.	
		" "	1 colony near tissue.	
		Spleen "	2 colonies, avoiding tissue.	
		" "	No "	
		Agar plate.	2 "	
92B	Strain 3	Heart tissue.	Numerous colonies, scattered.	
		" "	" " clustered about tissue.	
		Spleen "	No colonies.	
		" "	80 " mostly avoiding tissue.	
		Agar plate.	196 "	
93B	" 4	Heart tissue.	5 " clustered around tissue.	
		" "	10 " " " "	
		Spleen "	12 " avoiding tissue.	
		" "	8 " " "	
		Agar plate.	16 "	
94B	" 5	Heart tissue.	14 " clustered around tissue.	
		" "	21 " " " "	
		Spleen "	No "	
		" "	" "	
		Agar plate.	56 "	
96B	" 7	Heart tissue.	34 " " and scattered.	
		" "	44 " " all but 3.	
		Spleen "	18 " avoiding tissue.	
		" "	4 "	
		Agar plate.	140 "	
97B	" 8	Heart tissue, 5 day embryo.		65 colonies, scattered.
		" " " " "		Numerous colonies, clustered and scattered.
		Agar plate.		76 colonies.
99B	" 10	Heart tissue " " "		66 " clustered.
		" " " " "		94 " " about tissue and scattered.
		Agar plate.		110 colonies.

In this series larger cultures on glass box lids were used to bring out more clearly the influence of splenic tissue cells.

TABLE V.

Series 15. February 3, 1915. Fresh Plasma. 14 Day Chick Embryo Tissue.

Bacterium diphtheriticum inoculations. All cultures from 1st dilution.

106P	Strain 1	Heart, spleen, and liver tissue. " " " " " Agar plate.	No colonies. " " 352 "
107P	" 3	Heart and liver tissue. " " " " Agar plate.	No " Many colonies, scattered and clustered. 1,300 colonies.
108P	" 4	Heart, spleen, and liver tissue. " " " " " Agar plate.	Many " , clustered and scattered. Many colonies, clustered and scattered. 5,000 colonies.
109P	" 5	Heart tissue. " " Agar plate.	2 colonies near tissue. 26 " clustered and scattered. 140 colonies.
110P	" 6	Heart tissue. " " Agar plate.	1 colony near tissue. No colonies. 700 "
111P	" 7	Heart tissue. " " Agar plate.	No " " " 1,000 "
112P	" 8	Heart tissue. " " Agar plate.	Many colonies, clustered and scattered. Many colonies, scattered. 3,000 "
113P	" 10	Heart tissue. " " Agar plate.	50 " clustered. 179 " " 5,000 "

tissue per culture was not proportionably increased. Table I, No. 40, shows almost complete destruction of organisms in the presence of splenic tissue and plasma, and complete destruction with heart tissue and with plasma without tissue. However, here the cultures

must have contained very few organisms, as none developed when transfers were made to agar plates. In the small cultures of Table III the influence of dilution is seen in the scattering of the colonies, the organisms in some cases being able to overcome the influence of plasma even without the aid of tissue, but the larger cultures of Tables IV and V show more characteristic groupings and a marked destruction of bacteria, as seen by comparison of the plasma cultures with the agar plate counts. This destruction becomes more marked the larger the cultures, as is seen in Table V. All show the bactericidal action evident in the immediate vicinity of splenic fragments with the characteristic clear bacteria-free halos around the tissue fragments. This action is pronounced with *Micrococcus aureus* cultures, as previously noted, and is also seen at times with *Bacillus typhosus*, as in Table I, No. 43 A.

Influence of Tissue on Bacteria.

In addition to the bactericidal influence of growing splenic tissue noted above, which seems to be nearly coextensive with the area of migration of white cells in the plasma, and which must be due to such cells or their products, Tables I and II show another interesting action of tissue. These tables show that even where this bacteria-free halo is evident, often with *Bacillus typhosus*, at times with *Bacterium diphtheriticum* and with *Bacillus dysenteriæ*, a greater number of organisms may survive in the presence of splenic tissue than in heart cultures, and almost always more in the presence of any tissue than in plasma without tissue. This seems to show that the bacteria are either encouraged by the tissue or tissue products directly or are protected by them from the lytic action of the plasma. The spleen thus seems to have a double action, antagonistic in the immediate area of white cell migration and favorable in the area just beyond, probably due to cell secretions or excretions diffusing out from the tissue. This same double action of splenic cultures will be brought out in connection with tubercle bacillus cultures in a subsequent paper.

SUMMARY.

The results here reported confirm those of the former papers and strengthen the conclusions drawn therefrom. They may be summarized as follows:

Chicken plasma has a marked bactericidal action on *Bacillus typhosus*, which may be in some slight degree overcome by the presence of growing tissue, especially splenic tissue, in the cultures.

On *Bacillus dysenteriæ* this bactericidal action of chicken plasma is present, but much less marked, and the same counteracting action of tissue, especially splenic tissue, is evident.

On *Bacillus coli verus* chicken plasma has little or no bactericidal action.

On *Bacterium diphtheriticum* chicken plasma has a very strong bactericidal action which may be strongly counteracted by the presence of growing tissue in the cultures.

In all cases the bactericidal action of the plasma is decidedly diminished by dilution, as shown by the comparative results of these and the cultures formerly reported.

The migrating white cells from splenic cultures, or substances closely associated with these cells, have a distinctly bactericidal influence on all organisms tested except *Bacillus coli verus*. Murphy[3] states that lymphocytes first appear in the general circulation of the chick embryo on the 18th to 20th day, but in my cultures of splenic tissue cells resembling lymphocytes in morphology and behavior begin to appear in cultures of 11 or 12 day spleen and are abundant in cultures of spleen from 14 day or older embryos.

[3] Murphy, Jas. B., *Jour. Exper. Med.*, 1914, xix. 181.

THE REACTIONS BETWEEN BACTERIA AND ANIMAL TISSUES UNDER CONDITIONS OF ARTIFICIAL CULTIVATION.

III. The Action of Bacterial Vaccines on Tissue Cultures in Vitro.

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(Received for publication, October 14, 1915.)

While studying the action of pathogenic bacteria on chick embryo tissues cultivated *in vitro* by the method of Burrows,[1] the author was impressed by the apparent stimulation of the cultures inoculated with *Bacillus typhosus* when the bacilli themselves failed to develop. To determine if this was an actual stimulation of growth or merely an accidental condition in the few cultures so far tested, heavy vaccines, or suspensions of killed organisms, were prepared and used for diluting the plasma for tissue cultures, thus allowing the use of many more bacilli per tissue culture than could be used with living organisms. In moderate numbers, up to many thousands per drop, typhoid bacilli will not develop in chicken plasma cultures,[2] but by increasing the number of bacilli to the hundred thousands a few will develop in 50 per cent plasma. In the vaccine cultures, however, suspensions containing one billion or more bacilli per cc. were employed.

The author has previously reported[3] some preliminary results with the use of vaccines of *Bacillus typhosus* as well as of several other organisms with tissue cultures, but at that time no definite measurements were made for comparison with normal controls. Since then a number of other cultures have been made, and in each series estimates as accurate as possible have been made of the extent of new

[1] Smyth, H. F., *Jour. Exper. Med.*, 1915, xxi, 103.

[2] Smyth, H. F., *Jour. Exper. Med.*, 1916, xxiii, 265.

[3] Smyth, H. F., *Centralbl. f. Bacteriol., Ite Abt., Orig.*, 1915, lxxvi, 12.

growth in normal controls with plasma diluted with sterile Ringer solution and of cultures in plasma with equal amounts of the various vaccines suspended in Ringer solution. The latter results in general confirm those previously reported, except in the case of *Bacterium diphtheriticum* which in the preliminary tests seemed to have no definite action on tissue growth, but in the later more accurate tests proved to be somewhat stimulating to tissue cell growth although not so much so as were either *Bacillus typhosus* or *Bacillus coli verus*.

In estimating the extent of new growth in some cases three factors were considered, as in Tables I and II, and in others only two, as in Table III. The first figure given represents the mean diameter of the culture expressed in terms of that of the original fragment, the second figure is determined from the scale on the fine adjustment of the microscope, each unit representing ten points in the distance between the clearest focal points of the top and bottom of the cultures, while the third figure is an arbitrary one based on the observer's judgment as to the compactness of the new cells at the mid-focal point. From the results of many comparative measurements it would seem that as accurate determinations can be made by omitting the measurement of depth and considering only the extent of growth and density of cells as was done in Table III. These tables are given to show the method by which the results here reported were obtained, and they represent only a few of the cultures made. Table I shows results with vaccines of *Bacillus typhosus* and *Micrococcus aureus*, the temperatures at which the vaccines were prepared being stated in each case. All vaccines were from 24 hour agar slants. In Table II *Micrococcus aureus* and two strains of *Bacillus typhosus* were employed: W-J an old laboratory strain of Wyatt-Johnson, and A1 a recently isolated strain from a clinical typhoid case. In this table vaccines killed at low temperatures were compared with others killed at 100° C. In Table IV, in addition to the organisms employed in Tables I and III, vaccines of *Bacterium diphtheriticum* were used. As these were from fresh agar cultures they were of course free from toxin.

Table IV gives a summary of all the results of these series, except a few cultures of nervous tissue (cerebral vesicles and cord) which

TABLE I.

Series 5. October 29, 1914. Fresh Plasma. 12 Day Chick Embryo Tissue. Measurements Taken after 2 Days.

		diameter	*depth*	*density*	*total volume*	
Plasma + 56° C. vaccine, *B. typhosus*, Strain W-J.						
28s	Heart tissue.	12	2.5	3	−90	
	" "	9	3	2	−54	Average 66
	" "	6	3	3	−54	
	Spleen "	8	2.5	3	−60	
	" "	9	2	2	−36	" 40
	" "	6	2	2	−24	
Plasma + 60° C. vaccine, *B. typhosus*, Strain W-J.						
29s	Heart tissue.	7	2.5	2	−35	
	" "	5.5	3.5	2	−38.5	Average 36.75
	Spleen "	6	2.5	2	−30	
	" "	8	2.5	2	−40	" 33.3
	" "	5	3	2	−30	
Plasma + 60° C. vaccine, *M. aureus*.						
30s	Heart tissue.	3.5	1	2	− 7	
	" "	7	2	2	−28	Average 17.6
	" "	4.5	2	2	−18	
	Spleen "	5	1.5	2	−15	
	" "	6	2.5	3	−45	" 35
	" "	6	2.5	3	−45	
Plasma + sterile Ringer solution.						
31s	Heart tissue.	6	3	2	−36	
	" "	6	2	2	−24	Average 23
	" "	3	1.5	2	− 9	
	Spleen "	6	2	2	−24	" 24
	" "	6	2	2	−24	

Aureus vaccine depresses heart tissue, but stimulates spleen tissue.
Typhosus vaccine stimulates " " and " " "

TABLE II.

Series 6. November 4, 1914. Fresh Plasma. 14 Day Chick Embryo Tissue. Measurements Taken after 2 Days. Vaccines All Standardized 1,000,000,000 Bacteria per Cc.

		diameter	*depth*	*density*	*total volume*	
Plasma + sterile Ringer solution.						
32s	Spleen tissue.	3	x 2	x 3	−18	Average 21
	" "	6	x 2	x 2	−24	
Plasma + 56° C. vaccine, *M. aureus*.						
33s	Spleen tissue.	5	x 2	x 2	−20	
	" "	5.5	x 2	x 2	−22	Average 22
	" "	4	x 2	x 3	−24	
Plasma + 100° C. vaccine, *M. aureus*.						
34s	Spleen tissue.	3.5	x 2	x 3	−21	
	" "	3	x 2	x 3	−18	Average 17
	" "	4	x 1.5	x 2	−12	
Plasma + 56° C. vaccine, *B. typhosus*, Strain W-J.						
36s	Spleen tissue.	7	x 2.5	x 2	−35	
	" "	5	x 2	x 3	−30	Average 31.6
	" "	5	x 2	x 3	−30	
Plasma + 100° C. vaccine, *B. typhosus*, Strain W-J.						
37s	Spleen tissue.	7	x 2.5	x 2	−35	Average 30
	" "	5	x 2.5	x 2	−25	
Plasma + 56° C. vaccine, *B. typhosus*, Strain A1.						
38s	Spleen tissue.	6	x 2.5	x 3	−45	
	" "	4	x 3	x 3	−36	Average 33
	" "	3	x 2	x 3	−18	
Plasma + 100° C. vaccine, *B. typhosus*, Strain A1.						
39s	Spleen tissue.	4	x 2	x 4	−32	
	" "	7	x 2.5	x 3	−52.5	Average 40.16
	" "	8	x 1.5	x 3	−36	

Aureus vaccine at 100° C. depresses spleen tissue.
" " " 56° C. slightly stimulates spleen tissue.
Typhosus " " 100° C. stimulates spleen tissue.
" " " 56° C. " " "

TABLE III.

Series 12. January 10, 1915. Fresh Plasma. 12 Day Chick Embryo Tissue. Measurements Taken after 4 Days; No Account Taken of Depth.

		diameter		*density*	*total volume*	
Plasma + sterile Ringer solution.						
75s	Heart tissue.	2.5	x	2	−5	
	" "	3.5	x	1	−3.5	Average 5.2
	" "	3.5	x	2	−7	
	Spleen "	3	x	2	−6	
	" "	3	x	2	−6	" 5.3
	" "	2	x	2	−4	
Plasma + 100° C. vaccine, *B. typhosus*, Strain W-J.						
77s	Heart tissue.	5	x	2	−10	
	" "	8	x	2	−16	Average 13
	Spleen "	3	x	2	− 6	
	" "	4	x	2	− 8	" 8
	" "	5	x	2	−10	
Plasma + 60° C. vaccine, *M. aureus*.						
78s	Heart tissue.	1.25	x	1	−1.25	
	" "	2.5	x	3	−7.5	Average 4.37
	Spleen "	1.5	x	3	−4.5	
	" "	2	x	2	−4	" 4.25
Plasma + 100° C. vaccine, *M. aureus*.						
79s	Heart tissue.	4	x	2	−8	
	" "	1.25	x	2	−2.5	Average 5.25
	Spleen "	2.5	x	3	−7.5	
	" "	3	x	2	−6	" 6.5
	" "	3	x	2	−6	
Plasma + 100° C. vaccine, *B. coli verus*.						
80s	Heart tissue.	5	x	2	−10	
	" "	6	x	2	−12	Average 9.3
	" "	3	x	2	− 6	
Plasma + 1,000° C. vaccine, *B. coli verus*.						
80s	Spleen tissue.	4	x	2	−8	
	" "	3	x	2	−6	Average 7.3
	" "	4	x	2	−8	

Typhosus vaccine stimulates heart and spleen tissue.
Aureus " at 60° C. depresses heart and spleen tissue.
" " at 100° C. does not depress heart, and stimulates spleen tissue.
Coli verus " at 100° C. stimulates heart and spleen tissue.
The totals are small in this series as only two measurements were considered.

showed no definite results. Accurate comparative determinations of the amount of growth would be difficult to make, however, with these tissues as their growth is very irregular and varied in character under perfectly normal conditions. The vaccines tabulated as old were kept in sealed glass tubes at room temperature for over 6 months. The results with these old vaccines should not be given too much weight, as only one series of cultures was made with each, except in the case of *Micrococcus aureus* where two series were made of each. In this case it will be noted that whereas with the fresh 60° vaccines splenic tissue seems to be somewhat inhibited, it seems to be stimulated when the vaccine is heated to 100° C. or is kept for some months. The action of *Micrococcus aureus* vaccine on growth from heart tissue is variable, but never so actively stimulating as that of the typhoid or colon organisms, and at times is decidedly inhibitory. This inhibitory action when present is more pronounced than would appear from this table. The results of Table IV may be summarized as follows: *Bacillus typhosus* vaccines stimulate cell growth under all conditions. *Bacillus coli verus* vaccines when freshly prepared actively stimulate cell growth. *Bacterium diphtheriticum* vaccines mildly stimulate cell growth under all conditions. *Micrococcus aureus* vaccines seldom stimulate and usually inhibit cell growth when freshly prepared. Old vaccines lose this inhibitory action and seem to stimulate growth.

SUMMARY AND CONCLUSIONS.

These results seem to indicate that many bacteria may be utilized by tissue cells as food for growth or may contain a substance or substances stimulating cell growth or multiplication. This substance is stable and is not destroyed by heating to 100° C. or by long standing.

With *Micrococcus aureus* this action is often neutralized or overcome by a substance inhibitory to growth.

The nature of these substances has not yet been determined, though several attempts along this line were made by endeavoring to split the typhoid bacterial substance according to the method of Vaughan[4]

[4] Vaughan, V. C., Vaughan, V. C., Jr., and Vaughan, J. W., Protein Split Products in Relation to Immunity and Disease, Philadelphia and New York, 1913.

TABLE IV.

Summary of Vaccine Cultures with Tissue.

		Heart tissue.					Spleen tissue.				
B. typhosus.	56°	6 series, 14 cultures				+	4 series, 12 cultures				+
	60°	4	"	10	"	+	5	"	13	"	+
		1	"	2	"	0					
		1	"	6	"	−					
	100°	4	"	12	"	+	5	"	15	"	+
		1	"	7	"	−					
" " old.	100°	1	"	3	"	−	1	"	3	"	+
B. coli verus.	60°	1	"	3	"	+	1	"	3	"	− (all old).
	100°	2	"	7	"	+	2	"	5	"	+
" " " old.	10°	1	"	2	"	+	1	"	3	"	−
M. aureus.	56°	1	"	3	"	+	1	"	3	"	+
	60°	5	"	17	"	−	2	"	5	"	−
							2	"	6	"	+
	100°	2	"	5	"	+	2	"	5	"	+
		1	"	2	"	0					
		1	"	3	"	−	2	"	5	"	−
" " old.	60°	2	"	6	"	+	2	"	6	"	+
	100°	1	"	3	"	+	2	"	6	"	+
		1	"	3	"	−					
Bacterium diphtheriticum.	60°	3	"	6	"	+	3	"	9	"	+
" " old.	60°	1	"	3	"	−	1	"	3	"	+
	100°	1	"	3	"	+	1	"	3	"	+

+ = stimulation; 0 = no action; − = depression.

and testing the poisonous and non-poisonous residues separately. However, at the time too little bacterial substance was used to obtain enough end-products to be of much use, and the products so obtained prevented the plasma from coagulating. Even in the uncoagulated plasma there appeared to be an increase of cells in the cultures with the non-poisonous residue. The author hopes to be able to repeat these tests with split products at a later time when more bacterial substance is available, with the hope of obtaining more definite results.

THE REACTIONS BETWEEN BACTERIA AND ANIMAL TISSUES UNDER CONDITIONS OF ARTIFICIAL CULTIVATION.

IV. The Cultivation of Tubercle Bacilli with Animal Tissues in Vitro.

By HENRY FIELD SMYTH, M.D.

(From the Laboratory of Hygiene of the University of Pennsylvania, Philadelphia.)

Plates 42 to 47.

(Received for publication, October 14, 1915.)

The results obtained from the study of the reaction between various pathogenic bacteria and animal cells under conditions of artificial cultivation according to the method of Burrows,[1] led the author to undertake during the early part of 1915 the study of the action of human tubercle bacilli with chick embryo tissues cultivated in chicken plasma. Two strains of bacilli were employed in this work, one freshly isolated by the author from human sputum, using Petroff's egg-beef-juice-gentian-violet medium, and one obtained from the laboratories of the H. K. Mulford Co., a "Baldwin" bacillus used by them for preparing tuberculin.

Technique.

Inoculations were made from Petroff slants or from glycerine agar into sterile Ringer solution, and the inoculated saline was used for diluting the plasma (equal parts of plasma and saline) for cultures. In most cases the inoculated saline was simply shaken vigorously for a few moments to distribute the bacillary masses, but in a few instances it was shaken with sterile glass beads and then filtered through sterile glass-wool to remove all clumps. In each series a

[1] Smyth, H. F., *Jour. Exper. Med.*, 1915, xxi, 103; *Centralbl. f. Bacteriol., Ite Abt., Orig.*, 1915, lxxvi, 12.

number of cultures of the same tissues were made with and without bacilli, and specimens were fixed and stained at varying intervals after starting incubation.

Cultures were made on cover-glasses sealed on deep celled culture slides and also on the covers of glass boxes from one to two inches in diameter. All were sealed with soft paraffin and incubated at 38° C. Cultures were fixed in absolute alcohol, and when containing bacilli were stained for one or more hours with cold carbol-fuchsin, decolorized with acidified alcohol, and counterstained with Giemsa stain. This gave deep blue nuclei, faintly stained cell protoplasm, pink plasma, and clear bright red bacilli.

Colony Formation.

In these plasma cultures with or without tissue the tubercle bacilli develop freely with the formation of characteristic colonies. In specimens stained after three or more days there appear small, closely woven, filamentous colonies and frequently larger masses of irregularly twisted, loose, parallel threads forming an open network and resembling a tangled skein of spun silk. Where colonies encroach on tissue fragments these tangled skeins may be seen separating islands of tissue cells. In these colonies single organisms are hard to distinguish, being mostly arranged as solid or beaded threads resembling ordinary streptothrix colonies. Examples of this arrangement are shown in Figs. 1, 2, and 7. Fig. 1 also shows a compact colony, both colonies being shown under too low magnification to bring out single bacilli. Fig. 7 A, showing under the oil immersion lens a very small beginning colony, brings out the fact that the threads in these colonies stain irregularly, as do also streptothrix threads. This colony formation in loose skeins gives the picture described by Koch[2] but seldom seen in the solid media in general use at present.

Phagocytosis of Bacilli.

Phagocytosis of tubercle bacilli was clearly demonstrated in many cultures of splenic tissues and heart cultures (connective tissue cells)

[2] Koch, R., *Mitt. a.d. k. Gsndhtsamte.*, 1884, ii, 1.

as well as cells from skin and liver fragments. Fig. 2 shows new cells from heart tissue loaded with bacilli, Fig. 3 new cells from a fragment of epithelium from a four day embryo with a few bacilli, and Fig. 4 shows an epithelial plate from a liver fragment with a cluster of phagocytyzed bacilli. In all these cases careful focussing showed that the bacilli were within and not on the cells and they usually had a tendency to avoid the nucleus. In epithelial and connective tissue cultures the bacilli seem to have no especial attraction for the new cells, but are merely acted upon when the cells happen to come in contact with them in their wanderings, as all embryonal cells of whatever type are more or less actively motile in tissue cultures, or at least have motile processes.

Splenic Tissue Cultures.

The most interesting results, as would naturally be expected, were obtained from cultures of splenic tissue from fourteen day or older embryos. One series from a twelve day embryo, at which time there are few if any lymphocytes formed, gave the same picture as with heart tissue, occasional phagocytosis by connective tissue cells but no cell attraction. The most characteristic results were obtained with fourteen day splenic tissue, at which time, in cultures *in vitro* at least, lymphocytes or lymphocyte-like cells are very abundant and migrate freely over the entire field.[3] When a fairly heavy inoculation of unfiltered organisms with many small clumps is employed, the small round cells from the splenic tissue have a tendency to cluster around the clumps of bacilli as shown in Figs. 5 to 8. This clustering of lymphatic elements was observed as early as the third day with fourteen day tissue (Fig. 5) and within twenty-four hours in a culture from eighteen day spleen (see Fig. 8 B where small cells are seen on the surface of a large clump of bacilli). In Fig. 7 B is seen a loose cluster of bacilli and cells, in this case found floating in the serum from the culture in which were seen the other forms in the same sketch.

In all the series of cultures made by the author with tubercle bacilli the tendency of the new cells and migrating cells from splenic tissue

[3] Smyth, H. F., *Jour. Exper. Med.*, 1916, xxiii, 265.

to group in clumps or clusters throughout the plasma rather than to be evenly distributed was much more marked than in the normal bacteria-free controls, which of course were made with each series.

Giant Cell Formation.

Following the clustering of the lymphocytes around the bacilli the larger parenchymatous cells from the spleen, those of epithelioid type, approach, as shown in Fig. 5, and phagocytyze all bacilli with which they come in contact, seeming actively to seek out bacilli in a way the connective tissue and other cells do not. This is well seen in Fig. 6 B. These epithelioid cells containing bacilli, many of them with two or more nuclei, as is common in cultures *in vitro*, tend to fuse when they come in contact with each other as seen in Figs. 6 C, 8 A, and 9. In Fig. 9 A is a single mononuclear cell; B, B are cells with two nuclei and containing bacilli; C shows beginning fusion of three mononuclears; D of a binuclear with trinuclear cell; and E represents a completely fused multinuclear cell with bacilli. This process of fusion continues until by the seventh day many fully developed giant cells with numerous bladder-like nuclei arranged in characteristic groups, crescentic, bipolar, horseshoe-like, or circular, are seen. After this time these giant cells predominate, the smaller single cells gradually falling out of the plasma as it becomes liquefied by the cell ferments. Fig. 10 shows under a one inch objective an entire fragment of splenic tissue with degenerating reticulated center, radiating connective tissue growth, and many free giant cells scattered through the plasma. Figs. 11 and 12 are fully formed giant cells from a nine day culture, showing in Fig. 11 a cell with rounded margins embedded in deep plasma, and in Fig. 12 a surface cell spread out between plasma and cover-glass and having characteristic indistinct repand margins. The protoplasm in the center of these cells, particularly around the bacilli, appears degenerated and granular or structureless, while that around the nuclear groups is reticulate and healthy. Fig. 12 also shows in the small, dark blue masses the nuclear fragments of included lymphocytes.

In cultures up to three days many mitotic figures may be observed in the larger cells, but after the fusion of cells is fairly under way no

more figures are seen and they never appear in the fully formed giant cells. This supports the theory that tubercular giant cells are fusion cells, like Lambert's foreign body giant cells[4] and not due to the rapid division of nuclei without cell division. Evans, Bowman, and Winternitz[5] as the result of experiments with vitally stained rabbits, describe giant cells formed from epithelioid cells of the liver capillaries by nuclear division without cell division, but from their pictures the author believes that they correspond with the polynuclear cells seen in one to three day cultures as precursors of fully formed fusion giant cells.

Figs. 6 D and 7 C show what the author terms shadow cells, often seen in old cultures and interpreted as cells which having phagocytyzed bacilli are not able to digest them but are exhausted by the bacilli which grow in the cell, causing it to undergo degeneration until nothing but an indistinct shapeless mass remains. All stages of this process may be seen in cultures a week or more old.

Inoculations of Suspensions Free from Clumps.

The conditions noted above are those seen in cultures with heavy inoculations containing clumps of bacilli. Where the bacillary suspensions are first filtered to remove clumps these changes do not appear. In one series with a small 1 mm. loopful of turbid filtrate per 2 cc. of diluted plasma, and in another with three large 4 mm. loopfuls per 4 cc. of diluted plasma no bacilli developed in the neighborhood of either heart or spleen fragments, though a few small irregular acid-fast clumps were occasionally seen. However, these may have been artefacts due to deposits in the carbol-fuchsin. In another series made with a filtered culture and with a somewhat heavier inoculation, splenic cultures showed an occasional small loose cluster of bacilli with lymphocytes in cultures stained up to three days. In older cultures up to nine days no bacilli were observed within the areas of cell growth except at or near the limits of cell migration. In ten and eleven day cultures where the cells were degenerating, a few more bacilli were seen with one or two skein colonies, but still

[4] Lambert, R. A., *Jour. Exper. Med.*, 1912, xv, 510.

[5] Evans, H. M., Bowman, F. B., and Winternitz, M. C., *Jour. Exper. Med.*, 1914, xix, 283.

no bacilli near the tissue fragments or where new cells were plentiful. In a twelve day heart tissue culture from the same series the entire plasma was loaded with colonies and loose bacilli both near and away from tissue cells.

In another series using a bacillary suspension of the Baldwin type the suspension was shaken very vigorously with sterile glass beads before inoculation but was not filtered, and two and six drops respectively were added to 3 cc. of diluted plasma. Clusters of bacilli with lymphocytes were seen in twenty-four hour cultures, as well as epithelioid cells with phagocytyzed bacilli and a few large clumps of bacilli with lymphocytes adherent, as in Fig. 8 B. In eight day cultures a few giant cells were present, but not as many as with the freshly isolated strain of bacilli.

In cultures where the bacilli developed freely the splenic cultures contained more organisms than did those of other tissues, but in filtered cultures and light inoculations splenic cultures showed decidedly fewer organisms than did other tissue cultures. This agrees with results reported by the author with other organisms[3] where splenic tissue appeared to have a double influence: antagonistic, due to the action of the white blood cells or their products; and favorable, due to some other unrecognized cell substance or secretion.

The difference in action between cultures containing clumps of bacilli and those containing separated organisms only is one of mass action and corresponds to similar differences noted by Ayer[6] in his experiments on rabbits where unfiltered suspensions sprayed into the bronchi caused cavity formation while filtered suspensions did not.

SUMMARY.

In plasma cultures, with or without tissue, tubercle bacilli form characteristic streptothrix-like colonies of loosely twisted skeins of threads.

In plasma tissue cultures embryonal connective tissue and epithelial cells phagocytyze tubercle bacilli freely.

Splenic tissue cultures from fourteen day or older embryos if inoculated with isolated bacilli will phagocytyze and prevent the develop-

[6] Ayer, I., *Jour. Med. Research*, 1914, xxx, 141.

ment of all or nearly all bacilli present. If heavy inoculations of tubercle bacilli with many bacillary clumps are made in splenic cultures the bacilli develop more freely than in similar connective tissue cultures from heart tissue in spite of marked phagocytosis. In such cultures the first change seen is a clustering of small round cells, interpreted as lymphocytes, around bacillary clumps and colonies followed by a similar clustering of larger parenchymatous polygonal cells with one or several nuclei, interpreted as epithelioid cells, which phagocytyze any bacilli with which they may come in contact. The larger cells containing bacilli then fuse to form larger multinuclear cells and these by further fusion form typical giant cells with quiescent nuclei in characteristic groupings and bacilli in degenerated protoplasm away from the nuclear groups.

Cells which have phagocytyzed bacilli may digest them or may be unable to do so, in which case the bacilli develop within the cells and eventually cause complete cell degeneration.

CONCLUSIONS.

In plasma tissue cultures *in vitro* with tissue containing lymphatic elements the changes characteristic of early tubercle formation may be seen when such cultures contain masses of tubercle bacilli: First, clustering of lymphocytes about bacilli; second, clustering of epithelioid cells and other polynuclear or polymorphonuclear cells with multiplication of nuclei in the epithelioid cells; third, fusion of epithelioid cells to form giant cells; and fourth, degeneration of cells containing bacilli.

EXPLANATION OF PLATES.

PLATE 42.

FIG. 1. Tubercle bacillus colonies from 11 day culture from 14 day chick embryo spleen in chicken plasma inoculated with human tubercle bacilli, showing compact and loose skein colony. No tissue is shown. Drawn from Leitz oc. 4, obj. 6.

FIG. 2. Cells from 7 day culture of 10 day chick embryo heart tissue in chicken plasma inoculated with human tubercle bacilli, showing loose skein colony and new cells with phagocytyzed bacilli. Drawn from oil immersion lens $\frac{1}{12}$.

PLATE 43.

FIG. 3. New cells from 2 day culture of skin from 4 day chick embryo in chicken plasma inoculated with human tubercle bacilli, showing phagocytyzed bacilli. Drawn from $\frac{1}{12}$ oil immersion lens.

FIG. 4. Epithelial plate from 3 day culture of liver tissue from 7 day chick embryo in chicken plasma inoculated with human tubercle bacilli, showing phagocytyzed bacilli. Drawn from Leitz oc. 4, obj. 6.

PLATE 44.

FIG. 5. Cell clusters from 3 day culture of splenic tissue from 14 day chick embryo in plasma inoculated with human tubercle bacilli, showing clustering of small round cells around clumps of bacilli with a few larger parenchyma cells approaching. Drawn from Leitz oc. 4, obj. 6.

FIG. 6. Cells from 7 day culture of splenic tissue from 14 day chick embryo in plasma inoculated with human tubercle bacilli. A represents a loose cluster of tubercle bacilli and small round cells; B, a parenchyma cell with several nuclei approaching and phagocytyzing bacilli; C, a clump of fused parenchyma cells with phagocytyzed bacilli; D, shadow cells, degenerated cells containing bacilli. Drawn from Leitz oc. 4, obj. 6.

PLATE 45.

FIG. 7. Cells from 9 day culture of splenic tissue from 14 day chick embryo in chicken plasma inoculated with human tubercle bacilli. A represents a loose cluster of bacilli showing marked irregularity in staining; B, a cluster of bacilli and small round cells floating free in serum from culture; C, a shadow cell containing bacilli and a small amount of nuclear substance. B is drawn from Leitz oc. 4, obj. 6, and A and C are drawn from $\frac{1}{12}$ oil immersion.

FIG. 8. Cells from 1 day culture of splenic tissue from 18 day chick embryo in chicken plasma inoculated with human tubercle bacilli. A represents a group of small round cells and parenchyma cells, the latter having phagocytyzed some bacilli; B, a large clump of bacilli with small round cells adherent. Drawn from Leitz oc. 4, obj. 6.

PLATE 46.

FIG. 9. Cells from 7 day culture of splenic tissue from 14 day chick embryo in chicken plasma inoculated with human tubercle bacilli. A represents a parenchyma cell with a single nucleus; B, cells with two nuclei and phagocytyzed bacilli; C, fusion of three mononuclear cells with phagocytyzed bacilli; D, fusion of binuclear and trinuclear cells with bacilli; E, a small fusion multinuclear cell with bacilli. Drawn from $\frac{1}{12}$ oil immersion lens.

FIG. 10. 7 day culture of splenic tissue from 14 day chick embryo in chicken plasma inoculated with human tubercle bacilli, showing the original tissue frag-

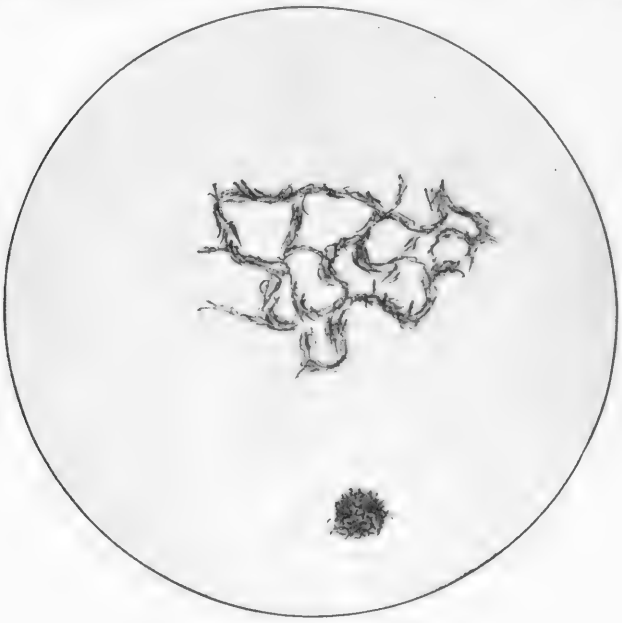

FIG. 1.

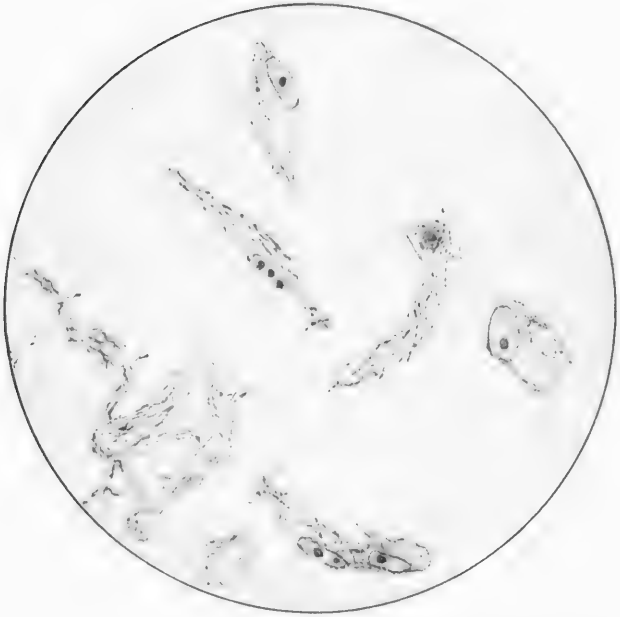

FIG. 2.

(Smyth: Bacteria and Animal Tissues. IV.)

FIG. 3.

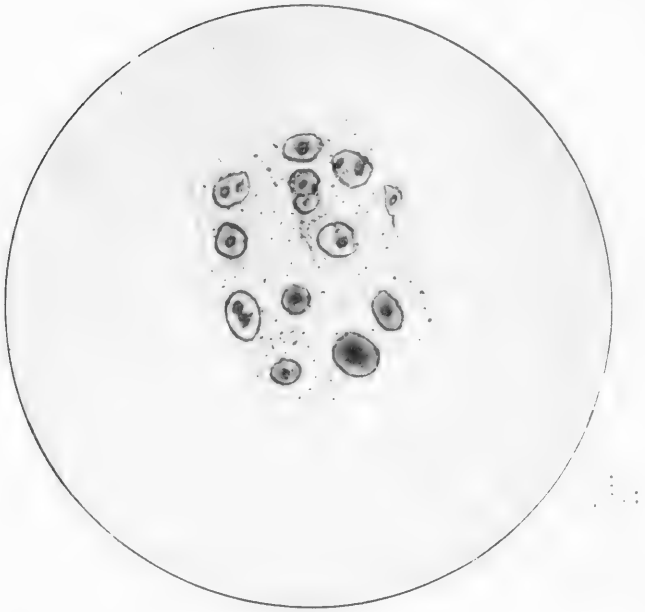

FIG. 4.

(Smyth: Bacteria and Animal Tissues. IV.)

FIG. 5.

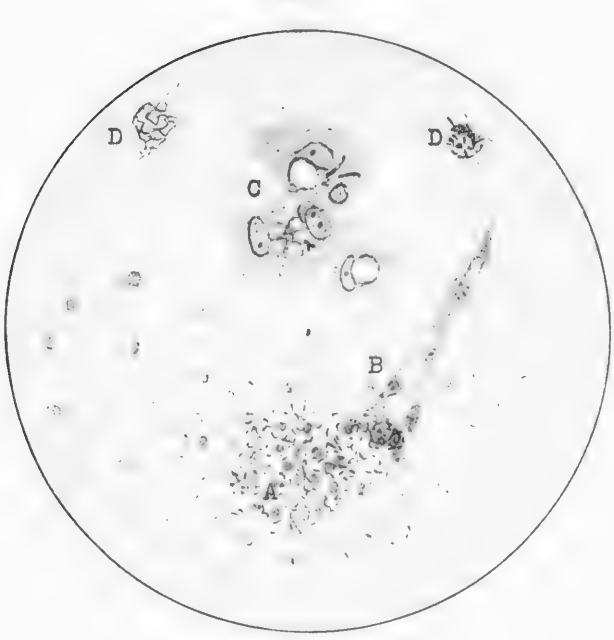

FIG. 6.

(Smyth: Bacteria and Animal Tissues. IV.)

FIG. 7.

FIG. 8.

(Smyth: Bacteria and Animal Tissues. IV.)

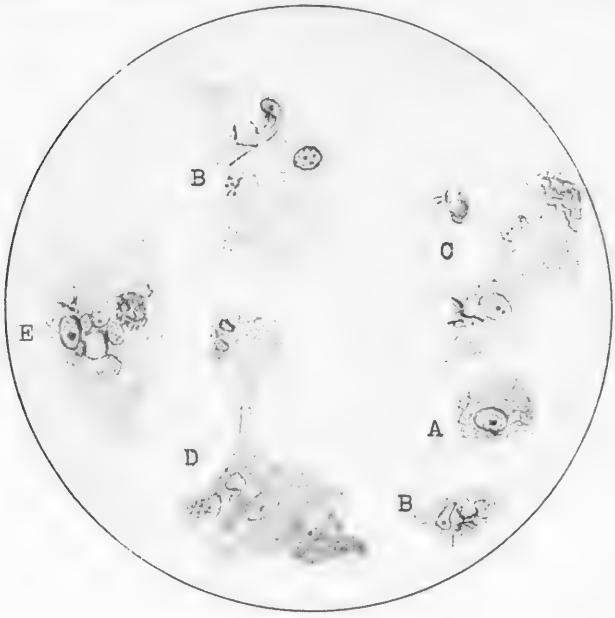

FIG. 9.

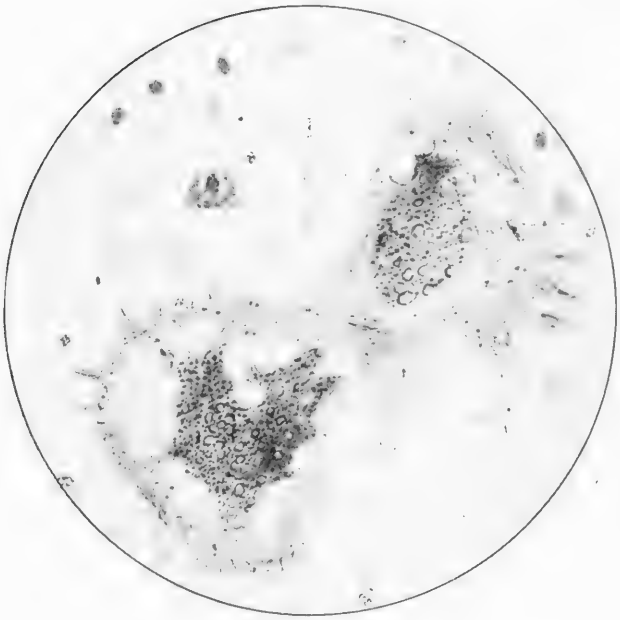

FIG. 10.

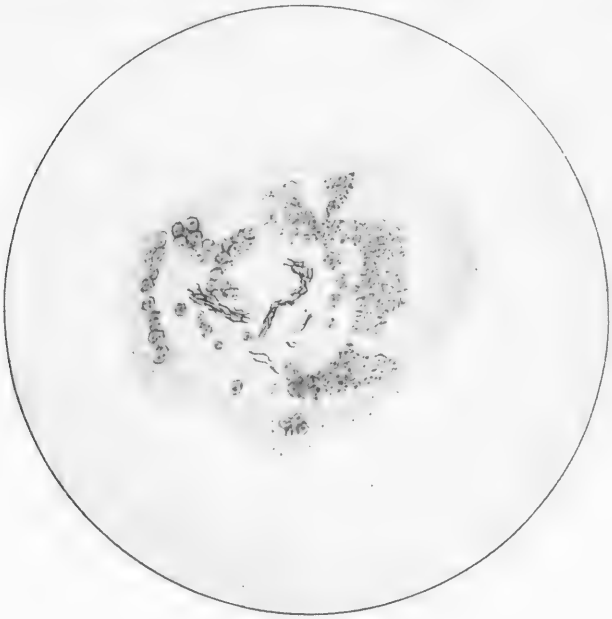

FIG. 11.

FIG. 12.

(Smyth: Bacteria and Animal Tissues. IV.)

ment becoming vacuolated, radiating new growth of connective tissue, and scattered large multinuclear and giant cells in plasma with clumps of bacilli. Small cells and single bacilli are not shown in this low magnification. Drawn from Leitz oc. 3, obj. 1 inch.

PLATE 47.

FIG. 11. Large giant cell from 9 day culture of splenic tissue from 14 day chick embryo in plasma inoculated with human tubercle bacilli, showing phagocytyzed bacilli. Drawn from Leitz oc. 4, obj. 6.

FIG. 12. Large giant cell from 9 day culture of splenic tissue from 14 day chick embryo in chicken plasma inoculated with human tubercle bacilli, showing phagocytyzed bacilli and fragments of small round cells. Drawn from Leitz oc. 4, obj. 6.

FIGS. 5, 6, 7, 9, 10, 11, and 12 are from the same series of cultures. Figs. 6, 9, and 10 are from the same slide; Figs. 7, 11, and 12 are also from one slide.

ABERRANT INTESTINAL PROTOZOAN PARASITES IN THE TURKEY.

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PLATE 48.

(Received for publication, January 11, 1916.)

During a study of tissues from a series of turkeys which had been included in several experiments designed to throw light on the transmission of the protozoan parasite producing the so called blackhead, the writer came upon a case which furnishes the text for this communication. The history of the animal (No. 98) is given in a recent paper (1).

It was raised with eighteen others in an incubator and brooder. No adult turkeys or poultry had been on the grounds for many years. Nine young turkeys from an infected flock, imported July 8, had been kept confined in a distant enclosure. Hatched about May 15, it was well until July 22, when it appeared quiet, sleepy, and refused food. It was chloroformed in the hope of obtaining fresh tissues for histological examination of the intestines. The autopsy showed an absence of blackhead lesions. There was, however, a distinct increase in mucus in the ceca, and several coccidial cysts, measuring about 24 μ by 17 μ, were found in the feces passed during chloroforming. The contents of the duodenum had a markedly yellowish coloration. The muscular tissue of the body was also tinged yellowish. Subsequent examination of tissues fixed in Zenker's fluid showed the presence of peculiar bodies in the subepithelial tissues of the villi of the small intestine, which were tentatively diagnosed as the asexual or schizogonic cycle of some coccidium. The apparently unusual position of the bodies in the adenoid tissue of the mucosa, where they were found in very large numbers, contrasted with the scarcity of the same within the epithelial layer where they, as will be shown

later, were probably not within but between the epithelial cells, induced the writer to give this case a more thorough study.

Coccidia have been generally regarded as exclusively parasites of epithelial cells. The older works of Balbiani (2), von Wasielewski (3), Doflein (4), and others take this for granted. Similarly the investigations by Malassez (5), Labbé (6), Pfeiffer (7), Sjöbring (8), Felsenthal and Stamm (9), Laveran (10), von Wasielewski (11), dealing either with *Eimeria stiedæ* of rabbits or the two known species of avian coccidia (*Eimeria avium* and *Isospora lacazei*) refer only to the epithelial cell parasites.

Metzner (12) was the first to call attention to the presence of coccidia in the subepithelial tissues. He "frequently observed the penetration of *Coccidium cuniculi* into the submucous tissue,[1] regularly into the tunica propria of the cecum, the appendix, and the colon, not infrequently of the small intestine." Metzner promised a discussion of these facts in a subsequent paper which has not, however, appeared.

Fantham (13) mentions the finding of coccidia of grouse in the submucosa, though "much more rarely." He further states that "active schizogony and sporogony go on in the ceca, chiefly in the epithelium, very rarely in the submucosa."

The writer (14), in 1910, described the occasional dislocation of sporonts or gametes of *Eimeria stiedæ* in the rabbit from the epithelial into the subepithelial tissue. This dislocation was ascribed to the transformation of invaded epithelial cells into multinucleated cells followed by disturbance of normal relationship with the adjacent epithelial cells and the subjacent tunica propria. The parasites were not found in the cells of the latter.

Hadley (15) refers to the invasion of the subepithelial tissues of the ceca of turkeys by coccidia. At that time he identified the protozoan parasite of blackhead, which is a purely connective tissue parasite and which does not enter epithelial cells at any time, as a coccidium.

Gérard (16) in describing a coccidiosis of young chicks takes an advanced position on the basis of material studied by him. He states that the schizogonic stage of his parasite goes on in the epithelial and subepithelial tissues and that sporogony goes on only in the epithelium. One of his figures shows many schizonts apparently in the subepithelial cells, but the drawing is evidently not quite true to nature and the large host cells, in certain cases at least, suggest epithelial cells. However, there seems to be enough evidence in his case to indicate extensive

[1] There seems to be much confusion in the use of the terms mucous and submucous tissue. The writer includes in the mucosa the tissue bounded by the muscularis mucosa. Several authors to be quoted have evidently regarded as submucous the subepithelial portion of the mucosa. Metzner in using tunica propria and submucosa as synonymous is using the latter term in this sense.

invasion of the mucous membrane with a slight simultaneous occupation of the epithelium.

It should be stated here that Rivolta (17), as early as 1873, described coccidia-like cysts in the submucosa of fowls which were relatively large and visible to the naked eye as white points, the size of poppy seeds. In 1878 he (18) again referred to similar white points about 0.5 mm. in size, in the same situation in fowls.

In 1893 the writer (19) published a brief account of similar cysts in the villi of the ileum in calves. These were situated near the free end of the villus, 0.3 to 0.4 mm. in diameter, and filled with mobile and immobile merozoites.

Two genera of sporozoa, very different from one another, yet both connecting the coccidia with the gregarinida, should here be mentioned, Léger and Duboscq's (20) *Selenococcidium* and Tyzzer's (21, 22) *Cryptosporidium*. In the former the schizont is a free-living nematode-like organism entering cells only during the periods of asexual and sexual reproduction. The latter passes its entire life cycle attached to, but not in epithelial cells.

Returning to the case of the young turkey, I wish at present to call attention to certain features only, for the sparse material and the absence of a sexual stage make any definite correlation with earlier work and any interpretation premature.

Sections of the intestinal tract were available from the upper, middle, and lower small intestine and the ceca. Conditions as regards the degree of invasion were practically the same throughout the small intestine, but over lymphoid tissue the parasites were scarce.

The epithelium was still present but lifted off from the subjacent core of the villus. The intervening space was filled with a network of circular lines of a precipitate made up of fine granules. The parasites were visible under a low power as vacuoles arranged in an almost continuous band near the margin of the villus core. Occasional bodies were nearer the central axis of the villus (Fig. 1). Under high powers these vacuole-like bodies were found to be but partially empty. A few were well filled. They consisted of some host cell whose cytoplasm had been moulded into a shell (or ring in section) with the much flattened nucleus against this shell. The contents were a very fine lining membrane within which were roundish bodies of various diameters, 2 μ and more, staining feebly reddish and with or without a mass of chromatin. Frequently a body contained two chromatin masses situated at opposite poles, as if division had taken place. Those bodies which were full of the small spheres, contained

about sixteen or more of more or less uniform size (Fig. 2). The vacuolated appearance under low power was due to the disappearance of some or all of the parasitic contents of the host cell. Prolonged search for the characteristic products of asexual multiplication—falciform bodies—brought to light only two or three parasites containing them. It is not to be denied that these may have been moulded into crescent shape by the pressure of the other growing and segmenting members in the same membrane. One of these crescents is shown in Fig. 3.

In addition to the parasites in the core of the villus, a certain number of bodies were found free in the space between villus core (tunica propria of Stoehr) and epithelium. If, as is claimed by most histologists, this space is an artefact, the free parasites must have been embedded in the adenoid tissue at the base of the epithelial cells and set free when the core was pulled away by the shrinking action of the fixing fluid. These bodies differ from those embedded in the core of the villus in having a more condensed cytoplasm, staining more intensely with eosin. They also contain relatively large and more numerous clumps of a substance staining deeply with nuclear dyes and presumably chromatin (Fig. 1 on the left above). These compact clumps are very irregularly grouped and of various shapes, many of them broadly oval or biconvex. No form which may be regarded as normal was detected, no two being precisely alike.

Relatively few parasites were found embedded in the epithelial layer itself. In the one shown in Fig. 2 there is considerable chromatin flattened against the periphery of the body as if it were a host cell nucleus. If so, the body is in a cell lodged between epithelial cells and not in the epithelial cell itself.

The same parasites were also found, but in relatively small numbers, in the mucous membrane of the ceca.

Assuming for the moment that all the parasites were striving toward the formation of merozoites of characteristic form, the great variation in the size of the products of division and the irregularity as regards chromatin, lead the writer to infer that they were largely degenerating forms. This theory is supported by the abortive attempts at repeated multiplication within the primary cysts and the partial disappearance of their contents.

As stated above, it is impossible from this case alone to determine whether the parasite belongs to one or the other of the two well known species of avian coccidia, or whether it is a foreign, aberrant type which fails to survive in the accidental host. The relative smallness of the schizonts, which measure 10 microns, and of the merozoites (the only one that could be found and measured being 5 μ long) led the writer to assume that it is a foreign species. The species described by Gérard has schizonts up to 30 μ by 42 μ, and merozoites 14 μ long (16).

The location of this parasite within cells of the villus indicates that the former may have actively invaded the cells and that phagocytosis did not play a part. The nature of the cells invaded the writer is unable to state.

In general, the invasion of the substance of the villus is of more than ordinary interest to the pathologist. A relatively large amount of foreign proteid is brought within the reach of the absorbents and the disintegration of the parasites may well account for the symptoms produced. Several other young turkeys died within a week after the case under consideration had been chloroformed, and the writer attributed these deaths to arsenic and lead spray. The symptoms in these cases were somnolence, indifference to food, and diarrhea, and the lesions were confined to hyperemia and swelling of the mucous membrane of the small intestines. Coccidia were not found in the intestinal contents or in scrapings of the mucous membrane. Unfortunately tissues of these cases were not saved for microscopic examination. Similarly there was slight temporary indisposition and drooping among other members of the flock, which may have been due to the unknown parasite. The presence of pheasants, sparrows, and song birds in the territory surrounding our experimental grounds may account for the infection, for the young turkeys were allowed to feed over a certain territory outside of the screened shelter each day (1).

Toward the end of the study of this case, the writer came upon two small areas in sections of the ceca of the same case within which nearly every epithelial cell contained minute protozoa which on further study were diagnosed as true coccidia (Fig. 4). The parasites were in an early stage, either as schizonts or sporonts. Putting all the

facts together, the writer is inclined to regard these epithelial cell parasites as belonging to a species distinct from that in the subepithelial tissue, and perhaps the same as the species represented by the oocysts found in the contents of the large intestine at autopsy. To all appearances we have in these parasites true coccidia to deal with.

The invasion of the subepithelial territory of the mucous membrane raises the question concerning the frequency of this phenomenon among coccidia. Is it that this early stage, coming as it does before or with the earliest symptoms, has been overlooked? The negative evidence the writer has cited above from the writings of others should not count too heavily, for much of the work was done by those for whom the life cycle of the parasite was the chief object of pursuit. It may now be desirable to examine the intestines of young healthy animals and of those in which symptoms are just appearing, to determine to what extent early schizogony goes on in the subepithelial tissues, whence direct invasion of epithelial cells for the sporogony may take place from the base of the cells.

There are so many points of difference between the blackhead parasite and the unknown parasite of the mucous membrane as encountered in this single case, that any attempt to present them would require an elaborate restatement of what is now published. Assuming that they are different, we are confronted with the fact that, even after the blackhead parasite shall have been eliminated, the outlook for raising turkeys without some losses due to avian coccidia and perhaps other still unknown protozoan parasites is not very encouraging. Fortunately the mortality due to these aberrant parasites was low. In any case the specific sources of coccidia and other parasites must be found and dealt with.

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EXPLANATION OF PLATE 48.

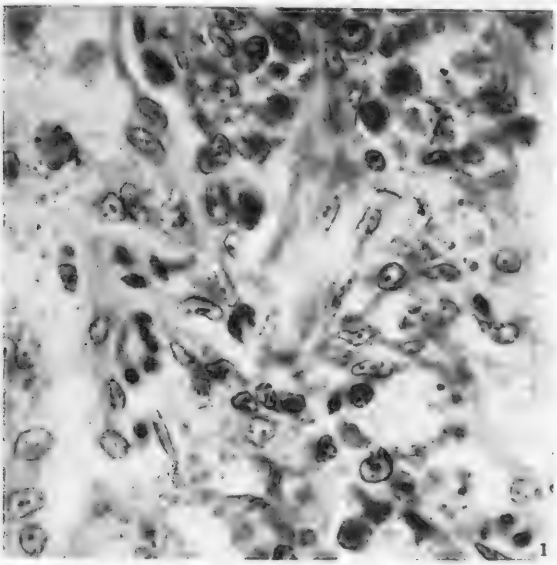
FIG. 1. Section through a villus. The main axis is from above down. Only a few nuclei of the epithelium appear on the left below. The core of the villus is surrounded by a bubbly coagulum under the epithelium. On the right, the marginal zone of the villus contains a row of faintly outlined parasites (from above down). The largest, below, appears as a vacuole containing a few remnants of the schizogonic division. On the left margin of the villus near the bottom of the figure several parasites are faintly outlined.

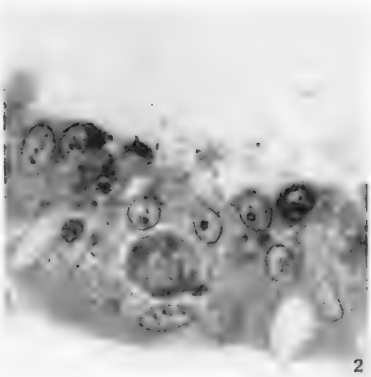
In the subepithelial (bubbly) space on the left, above, a parasite is seen staining more intensely and with six or more clumps of chromatin.

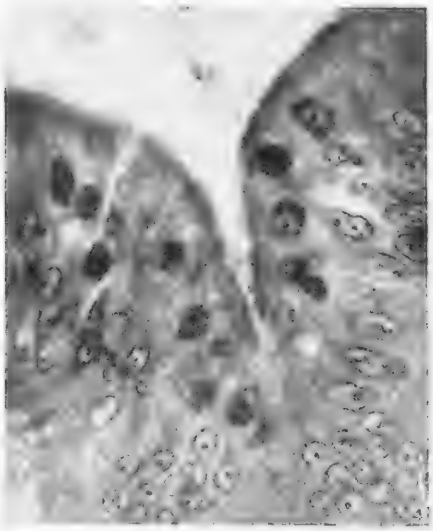
FIG. 2. A parasite within the epithelial layer. The nucleus of the host cell is shown only in a small part in the photograph. The nucleus of an epithelial cell is seen in the invaded territory crowded towards the free border of the epithelial layer. The progeny of the schizogonic (?) division are faintly outlined. Only one shows a nucleus indistinctly.

FIG. 3. A schizont showing what appears to be a merozoite with a nucleus near one extremity. The schizont is situated in the space between the epithelium and the villus core and is evidently attached to the latter. A row of parasites situated in the marginal zone of the villus is faintly indicated.

FIG. 4. Epithelium of one cecum, showing nuclei near the base of the cells and parasites nearer the free border. The parasite farthest to the right contains four nuclei, but only two are in focus.







(Smith: Aberrant Intestinal Protozoan Parasites.)

A FURTHER STUDY OF THE REGENERATED EPITHELIUM IN CHRONIC URANIUM NEPHRITIS. AN ANATOMICAL INVESTIGATION OF ITS FUNCTION.

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PLATES 49 TO 52.

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In a previous report (1) the writer has studied in some detail the changes which follow an acute attack of experimental uranium nephritis and called especial attention to the changes which occur in the epithelial structures of the diseased kidneys. It was pointed out that the regenerative processes differ in a marked degree from those observed following the acute degenerations of other toxic agents, in that the newly formed epithelial cells never assume, or even approximate, the structure of the original renal cells. The cause of this abnormal development was considered to be the deleterious effect of the developing connective tissue, which began at an early period and progressed continuously until the death of the animal from the resulting chronic nephropathy.

That this active proliferation of the connective tissue is lacking with the majority of renal toxic agents is well known, having been confirmed by many authors (Ophüls (2), Dickson (3), Suzuki (4)), and following such degenerations the process of repair follows a different course. Thorel (5) in his studies on regeneration following acute chromium nephritis has shown that there is practically a complete restitution of the degenerated epithelial cells after nine days, though a few tubules with a greater number of nuclei than that seen in the normal tubules may persist for a time in cases of severe damage. This, he states, is the only anatomical lesion persisting for any length of time.

In chronic uranium nephritis, on the other hand, it has been shown that the regenerated cells are easily picked out in the areas of fibrosis as late even as 104 days. The most prominent characteristic of these regenerated cells is the large vesicular nucleus, which is of an oval or irregular shape. A nucleolus is, as a rule, prominent and the chromatin threads are thick and stain heavily with hematoxylin. The "*Kern-Plasma*" relation shows a marked disturbance as compared to the original renal cell. The protoplasm is very scanty, in many cases forming so thin a covering band for the nucleus that it is difficult to see. In other examples, especially in the earlier stages of the process, giant cell-like complexes are seen with a comparatively large amount of protoplasm. This apparent excess of protoplasm is, however, distributed in the later stages over the membrana propria, and the thin covering that it then forms for the tubule is evident.

The question at once presents itself, whether these cells, which anatomically differ so markedly from the original renal cell, are able to functionate in a normal manner. A suggestion that their function is altered is found in the work of Ribbert (6), who has shown that the regenerated cells fail to show the carmine deposits after intravenous administration of the dye, which are seen in the original renal cells. Though these findings were made in the regenerated cells in early stages of repair, the writer has observed that even in the latest stages (54 days) of chronic uranium nephritis, the newly formed cells fail to show the deposits of the dye. Whether this (the presence of vitally stained granules in the cell) may be taken as evidence of secretion, will be considered at a later point of the discussion, though their absence certainly points to an abnormal functional state of some sort in the regenerated cells.

Anatomical changes and their relation to function in the kidney cells have interested observers since the beginning of microscopical investigations. Though no attempt will be made to cover the extensive literature of this subject, for which the reader is referred to monographs on the kidney (Policard (7)), attention is called to certain representative articles.

One group of observers has described vacuolar changes in the renal cells during periods of active secretion. Among these may be noted Gurwitz (8), Lamy, Mayer, and Rathery (9), Enesco (10), and others.

The greater number of investigators, however, have connected changes in the *batonnets* of Heidenhain with variations of function.

The most definite changes in these structures have been observed by Regaud and Policard (11) in the kidneys of the Ophidia. The cells of the kidneys of these animals show a marked variation in the nature of their content, especially in that part of the tubule corresponding with the proximal convoluted tubule of mammals. In some the cells are filled with large granules (*grains de ségrégation*), while in other tubules no granules, but rods are found. Intermediate stages are common. The batonnets in the former type of cell have almost disappeared and exist as short, sinuous filaments localized to the infranuclear zone of the cell, while in the latter forms, *i.e.*, without grains, they are much longer and stretch from the base to the apex of the cell. From the intermediate stages, in which the grains may be seen forming in the batonnets, the authors conclude that there is a direct transformation of the filaments to granules. The filaments, or batonnets, are therefore considered as the agents of elective intussusception for the introduction into the cell of the substances carried in the blood. Analogous findings have been described by Policard (12) in the Batrachians.

In mammals the changes are less definite. Takaki (13), studying the batonnets in the renal cells of the mouse, describes them in resting conditions as homogeneous rod-like formations, which in stages of secretion (feeding of fluids, diuretin administration) change to granules which still preserve the rod-like arrangement of the batonnet. Under pathological conditions somewhat similar changes are described at the outset, which ultimately result in the production of coarse granules of varying size and shape. Kolster (14) and his pupil Hjelt (15) have studied the rabbit kidney with similar result. Enesco finds the same appearances in caffeine and theobromine diuresis.

The greatest objection to the interpretation of all these descriptions of changes in the mitochondrial apparatus as indicative of a normal secretory process, is the fact that similar changes have been described in the study of degenerative processes, especially that known as cloudy swelling. The late stages of this condition, in which large irregular granules are produced, are easily recognized as patho-

logical, but the beginning formation of these granules from the batonnets is apparently identical with the changes described for the process of secretion (Landsteiner (16), Takaki). This fact has led many authors to consider the condition of cloudy swelling as a process of hypersecretion. A further consideration of this phase of the subject will be given in the discussion of the findings of the present investigation.

Though the mitochondrial apparatus of the normal kidney cells, both resting and secreting, and the changes which occur in them in acute degenerations have been carefully studied, so far as we know no descriptions have been given of these structures in the regenerated cells following experimental lesions. As changes in them have been connected with functional variation by so many authors, we have attempted in this investigation to describe their appearance in the regenerated epithelium of chronic uranium nephritis, and have repeated some of the experiments of previous writers on normal kidneys.

We wish to emphasize at this point that there is a marked anatomical difference in the regenerated epithelium in chronic uranium nephritis and those newly formed cells seen after acute experimental nephritides which are not followed by a progressive chronic nephritis (chromium, sublimate). The findings of the present investigation cannot be applied to the latter, as in them the anatomical restitution is complete. From what we know of studies of the acute degenerations in man, there is most likely an equal functional repair. Functional studies of experimental nephritis have been largely confined to the early stages of acute degeneration in which the animal died. Siegel (17) describes a case of uranium nephritis in a dog in which death did not occur for thirty days. In this experiment the secretion of the different urinary constituents decreased until death.

Variations in the Mitochondrial Elements of Normal Kidneys in Different Functional States.

In order to obtain specimens of kidneys in various stages of activity two methods were used. In one set of experiments white rats were given no food or water for three days. In these animals the urinary excretion ceased on the second day as a rule, and little or no urine was found in the bladder when the animal was killed. Another

group of rats was given 2.5 cc. of 5 per cent urea solution intraperitoneally. In some instances this dose was repeated in one hour. The animals as a rule urinated about one hour later, and were killed one hour after the last injection. The fluid in all cases was readily absorbed, and the bladder contained a large amount of urine. The kidneys were removed at once and small pieces fixed in Kolster's fluid which is based on the Regaud technique for showing mitochondrial structures. Thin paraffin sections (3 to 5 μ) were stained with iron-hematoxylin.

Appearance of the Batonnets in Periods of Rest.—In the kidneys of animals which had secreted little or no urine for one or two days, the batonnets presented a strikingly uniform character. In sections of the proximal convoluted tubule which pass at right angles to the lumen of the tubule, they stretch as long, somewhat sinuous rods, from a short distance from the basal membrane to a varying distance towards the lumen of the tubule. As the lumen is very narrow in the resting tubule, the batonnets never reach it, but end at a point about twice the diameter of the nucleus from the membrana propria. Where the section passes obliquely through the tubule, the batonnets are cut into short cylindrical rods, but never appear as isolated round granules.

The ascending limb of the loop of Henle and the distal convoluted tubule are readily distinguished from the above described segment by the relative shortness of their chondrioconts. In these divisions as well, the batonnets exist as homogeneous cylindrical formations, and show much less affinity for the iron-hematoxylin than those of the proximal convoluted tubule.

As it is impossible to place the kidney in a state of absolute rest, one can always find tubules which do not show these characteristic appearances. A few tubules are seen in which the batonnets resemble the descriptions given in the following section.

The Batonnets in Stages of Diuresis.—Though the most apparent variation in the kidneys of rats in stages of diuresis is the dilatation of the lumina of the tubules, certain constant changes are seen in the mitochondrial apparatus of the proximal convoluted tubule. Instead of the long homogeneous structures seen in the resting tubule, the batonnets are now distinctly granular. As well as these small cir-

cumscribed swellings in the rod, isolated granules are also seen, especially in the apical region of the cell. These granules are distinctly round, as compared to the rod-like cut sections of the batonnet seen in the resting tubule, and are of small size, their diameter being slightly greater than that of the original batonnet. The batonnets are, moreover, swollen and much thicker than in the resting kidney. The cell body as well shows this enlargement, the apex of the cell being especially swollen.

The rods of the ascending limb of the loop of Henle and of the second convoluted tubule show no change whatever, but resemble perfectly the appearances seen in the resting kidneys. The less well developed mitochondria of the collecting tubules are also unaffected.

In some instances, in which large amounts of normal salt solution were suddenly injected into the peritoneal cavity, changes were seen which resemble those described in pathological conditions. The droplets were large, even of the diameter of the nucleus, stained irregularly, and were scattered without orderly arrangement. Such appearances were never seen when moderate doses of injection fluid were used.

The Mitochondrial Apparatus in the Regenerated Cells of Chronic Uranium Nephritis.

The kidneys examined in these experiments were those of guinea pigs which had received 3 mg. of uranium and which had been killed at a time when the animal had recovered from the acute effects. Former experiments (1) have shown that the acute lesions are completely replaced by the regenerated cells by the fifteenth day, and as the newly formed connective tissue has not shrunk to any great extent by this time and so obscured the epithelial structures, such a period is most satisfactory for a detailed study of the structure of these cells. As a control other animals were allowed to live until death, one surviving to the eighty-fifth day.

The general structure of the kidney at the fifteenth day of the nephritis has been previously described. The degenerated cells have been entirely replaced by large, irregular epithelial cells, with large oval nuclei, and stand out prominently in the affected areas. The

connective tissue in these regions is still largely cellular, but the beginning collapse of the tubules gives evidence of its shrinkage. In the cortex the effect of this collapse is seen in the dilatation of the proximal convoluted tubule and the glomerular space. A few hyaline casts still persist in the tubules.

In sections stained with iron-hematoxylin after fixation in Kolster's fluid, one is at once struck by the scarcity of the mitochondrial elements as compared to the normal kidney (Fig. 1). With a low magnification the section resembles the appearances seen in vitally stained kidneys. Around each glomerulus is seen a group of tubules which contain the deeply stained batonnets, while the remainder of the cortex is almost entirely free of tubules which possess these heavily stained structures (Fig. 2).

Examination with a higher power shows that the deeply stained groups are Division I of the proximal convoluted tubule.[1] The cells of these divisions are entirely normal in every regard. As the animals had received urea solution intraperitoneally for a purpose to be described later, these tubules show the changes described above as characteristic of a secretory phase. The rodlets are granular and the lumina dilated. More widely scattered through the cortex are seen the distal convoluted tubules and the collecting tubules. These as well are entirely normal, though the latter commonly contain casts, and show no changes in their mitochondrial apparatus.

The most striking appearance, however, is found in the protoplasm of the regenerated cells. Nothing analogous to the batonnet which existed in the original renal cell is seen. In many of the regenerated cells an indefinitely arranged finely granular material is scattered throughout the protoplasm, but never do we see the definite cell organs such as were formed of the mitochondrial substance in the original cells (Fig. 3). In many cases it is difficult to decide if these

[1] The terminology is that of Suzuki, who divides the proximal convoluted tubule into Divisions I, II, and III, according to the amount of vital dye seen in them. Division III is the terminal, and extends into the medulla as far as the boundary of the outer and inner stripe of the outer zone of the medulla. It is this Division III which is affected by uranium and in which most of the reparative processes take place. For further details see Suzuki (4) or the writer's previous article (1).

irregular granules give the reaction of the mitochondria, as they stain so lightly and irregularly. In other cells these granules are more deeply stained, and can then be definitely classed as mitochondria.

Occasionally a tubule is seen which contains batonnets and at the same time evidences of nuclear proliferation. These are intact Divisions I and II of the proximal convoluted tubule, which, as has been described by the writer, often show proliferative tendencies.

The structure of the batonnets and the changes occurring in them in stages of diuresis, differ in no regard from the descriptions of Takaki, and are essentially the same as those figured by Kolster, Hjelt, and Enesco. As compared with the definite changes described in the lower animals, the findings in mammalian kidneys are much less satisfactory. This can perhaps be best explained according to Regaud (18) who says:

> We are therefore led to suppose that in the mammals the phenomena of concentration (in the cells of the tubules) are at a minimum, the morphokinetic variations less marked, and the functioning of the cells continuous, while in the Ophidia the characteristics of function are quite different, phenomena of concentration very important, functioning of the cells discontinuous, alternative and periodic, morphokinetic variations pronounced.

We cannot go into the details of the interesting theory of Regaud and others (Arnold) concerning the relation of the mitochondria (plasmosomes) to cell function, but must refer the reader to the original articles of these authors for the consideration of their deductions. There can be no doubt that the mitochondrial elements of certain divisions of the renal tubule (proximal convoluted tubule) show changes in periods of active diuresis, and that such changes are very limited in periods of comparative rest of the kidney, and are not found in other segments of the tubule.

The relation of these changes to the pathological condition of cloudy swelling is difficult to determine. The two extreme interpretations are, either that the state of cloudy swelling is a manifestation of hypersecretion, or that all changes in the batonnets which result in granule formation are pathological, and both these conceptions have been accepted by eminent investigators. Two facts argue strongly against the latter. First, it is not likely that the injection of a few

cc. of a non-toxic substance (urea or salt) would result in damage to the kidney. Secondly, the formation of granules from the batonnets in the Ophidia and Batrachians occurs without intervention of any sort. It is quite conceivable that the reaction of the cell to a normal functional stimulus and to a weak pathological stimulus may take the same morphological expression, and it is not necessary, in this discussion at least, to draw any wide-reaching conclusions from the scant evidence at hand.

The marked difference which the mitochondria of the regenerated cells in chronic uranium nephritis show in contrast to the original renal cells is a point of special interest. The mitochondria which they possess are similar to the findings in cells of a low degree of differentiation, for in such have been described great variation in amount as well as in the morphological character of these elements. The studies of Lewis and Lewis (19) of the mitochondria in tissue cultures show the wide variations in such proliferating cells. In like manner the mitochondria of the regenerated cell in the kidney vary widely in amount and never show the formation of definite cell organs, the batonnets. While in most epithelial degenerations in the kidney, the regenerated cells, though at first of this embryonic type, later assume a form which differs in no degree from the adult type of the original renal cells (Thorel), in chronic uranium nephritis the early proliferation of the connective tissue or some other factor apparently prevents these epithelial structures from reaching maturity.

We now come to the consideration of the function of these cells in chronic uranium nephritis. As mentioned in the introduction of this article, the absence of dye granules in these cells is suggestive of altered function, though the demonstration of the secretion of some substance native to the normal urine would be much more convincing.

Microchemical tests have been applied to the kidney by many authors, but satisfactory results have been but rarely obtained. Recently Leschke (20) has developed a technique which enables one to demonstrate a large proportion of the urinary constituents in the cells of the renal tubule. Briefly reviewed, he has shown that sodium chloride, urea, phosphates, and uric acid are only present in any practical degree in the cells of the proximal convoluted tubule, including its medullary segment. The glomeruli never show any deposits

of these substances. The finding of the urinary constituents in the cells of the tubules can only be interpreted as evidence of the secretion of these substances into the lumen of the tubule.[2]

This method of anatomically studying the function of the renal cells has been applied to the problem of the function of the regenerated cells in chronic uranium nephritis. As the technique of the various tests is somewhat difficult, urea was selected as representative and demonstrated in the kidneys of the same animals whose kidneys were described in the previous division in which the changes in the batonnets were discussed. We were thus able to observe the morphological changes in secretion by two different methods in the kidneys of a single animal, and so a means of comparison and added control was given.

The Secretion of Urea in the Normal Kidney.

As a confirmation of Leschke's work and as a control on the experiments on nephritic kidneys, several normal animals (guinea pigs) were given different doses of urea solution intraperitoneally. Leschke's technique was followed in detail. It is based on the old quantitative method of Liebig. The kidneys were fixed by injection in a strong solution of mercuric nitrate, washed, and the resulting urea-mercuric nitrate compound reduced by hydrogen sulphide in thin paraffin sections. The method of injection of the fixing solution directly into the artery we found much more satisfactory than fixation in block, as the reagent penetrates very poorly. When counterstained with hematoxylin, the resulting compound, which for convenience we shall refer to simply as urea, shows as a grayish green or brownish black color, depending on its concentration in the cells. As Leschke points out, all protein gives the reaction to a slight degree, but the contrast in the places of deposit of the urea and tissues where no such con-

[2] It is obvious that if one assumed that the substances present in cells of the tubule were absorbed from the lumen, then the concentration of the urine would be lowered by the process. The difficult point to explain by Ludwig's hypothesis has been the large amount of absorption of water necessary to raise the concentration of dilute glomerular urine, so that an assumption of an absorption of the solid constituents would further complicate this theory.

centration can exist (connective tissue) is always sufficient for practical purposes.[3]

The histological appearance of the kidneys of animals which were secreting relatively large amounts of urea, and which were prepared by the method described above, is remarkably constant. An examination of the section with a hand lens shows the divisions of the kidney to be sharply outlined, due to the varying amounts of urea which they contain. The cortex and outer stripe of the outer zone of the medulla are intensely dark, then, considerably lighter, the inner stripe of the outer zone, and finally the comparatively pale inner zone of the medulla.

With a higher magnification it is seen that the darkness of the cortex and the outer stripe of the outer zone of the medulla is due to the loading of the cells of the proximal convoluted tubule with dark granules. The details of the cells are best seen in kidneys where the secretion of urea is at a low concentration, as otherwise the dark granules obscure the finer structures. In such specimens the granules appear as small round bodies which extend from the base of the cell to the apex in definite rows, each granule being connected with its neighbor by a thread-like formation (Fig. 4). When the long axis of the cell is curved, these rows follow the curve of the cell. The apex of the cell shows a definite swelling in this stage, and its protoplasm gives evidence of beginning vacuolization. The cuticular seam is well preserved (Fig. 4).

In kidneys secreting a more concentrated urine the details are less clear. A large number of dark granules fills the entire cell body, obscuring even the nucleus (Fig. 5). The apices of the cells are greatly

[3] *In vitro* a precipitate is formed in the mercuric nitrate solution when sulphates or phosphates are added. These are, however, soluble in acid solution, so that one can prepare a solution of mercuric nitrate, acid with nitric acid, which will throw down the urea alone. As the amount of acid to be added varies with the concentration of mercuric nitrate in the fixative solution, it should be determined by experiment before each injection. Solutions of phosphates and sulphates of a concentration known to be slightly greater than that found in the urine, and a solution of urea roughly approximating that of urine, are prepared. The solution of mercuric nitrate is then acidified with strong nitric acid until the desired point is reached.

swollen and the cuticular seam is no longer evident. The lumen of the tubules contains precipitated masses, and the cut sections of the swollen apices of cells, whose bodies are out of the plane of the section. It is in such stages, in specimens whose fixation is not perfect, that the lumen is transformed into a network of irregular threads from the distortion of these fragile swollen cell apices, and the appearances noted by many authors (Suzuki) after certain fixatives (formalin) are obtained.

Scattered among these heavily stained proximal convoluted tubules are seen the lighter colored distal convoluted and collecting tubules. The latter never show any granules whatever of urea, and though their protoplasm, especially in the large ducts of Bellini, may be rather dark, it is always homogeneous. The distal convoluted tubule and the ascending limb of Henle's loop show a finely granular protoplasm. It was impossible to decide whether these were small granules of urea or not, but they never exist in such amount as to have much practical importance in the secretion of urea. Nor is there the marked variation in these granules depending on the concentration of the urea secretion in the urine, as is seen in the proximal convoluted tubule.

The location of the urea in the normal kidney is essentially that given by Leschke. It has, however, been claimed, in spite of the fairly complete controls which he gave, that the reaction has nothing to do with the urea secretion, but that there is some affinity on the part of the mercuric salt for the proximal convoluted tubule, as all who saw Leschke's preparations granted that the deposits were limited to all practical extent to this segment of the renal tubule. To meet this objection, we have, with the aid of Drs. Addis and Watanabe, combined the study of the urea concentration of the urine with the microchemical demonstration of urea in the renal cells. Guinea pigs were treated as shown in the protocol, the bladder urine was collected at autopsy, and the kidneys were prepared in the manner described above. The urea concentration of the urine was determined by the Marshall urease method by Drs. Addis and Watanabe. Table I gives the details of certain typical examples.

We see that the staining due to the deposit of mercuric salts occurs only in the proximal convoluted tubule and that the amount varies directly and proportionately with the amount of urea secreted in the urine. With these facts determined, we can proceed to the study of the function of the regenerated cells in chronic uranium nephritis.

TABLE I.

Treatment.	Amount of bladder urine.	Amount of urea.	Urea.	Histological appearance of kidney (urea).
	cc.	*gm.*	*per cent*	
Guinea Pig 12. Usual food preceding experiment. 10 cc. 20 per cent urea solution intraperitoneally. 1 hour later killed and bladder urine saved.....	7.7	0.2152	2.79	Very large amounts of urea in proximal convoluted tubule; all details in cells obscured.
Guinea Pig 10. Three days of green food. Water intraperitoneally and killed 1 hour later..	5.0	0.0837	1.674	Moderate amount of urea in proximal convoluted tubule, these plainly darker than ducts of Bellini.
Guinea Pig 11. Same as guinea pig 10....	3.0	0.0179	0.596	Very small amount of urea. Difficult to make out difference in color between proximal convoluted tubule and ducts of Bellini.

The Secretion of Urea by the Regenerated Epithelium in Chronic Uranium Nephritis.

The kidneys examined by Leschke's method were the same as those described above in the study of the changes occurring in the batonnets in chronic uranium nephritis.

With the low power the areas of regenerated cells and fibrosis stand out in the darkly stained cortex, in the medullary rays, and outer stripe of the outer zone of the medulla, as lighter patches (Fig. 6). On more detailed examination the normal Divisions I of the proximal convoluted tubules are found to contain large amounts of urea, and differ in no way from these same segments of the tubule in normal kidneys. The large regenerated cells, however, show a striking difference from the original renal cells in that they show little or no evidence of the urea granules. In some cells, it is true, a few dark isolated granules are seen, but never in a degree even approaching that found in the persisting convoluted tubules. The protoplasm, in the majority of cases, is extremely clear and transparent (Fig. 7). Many of the tubules show collapse, and in these the absence of urea granules is particularly striking. In other tubules the giant cell-like complexes are seen and their protoplasm as well contains no granules. In the medullary rays the persisting segments of the proximal convoluted tubule stand out in marked contrast, as their protoplasm contains the urea in large amount, to the destroyed and regenerated terminal divisions of the proximal convoluted tubule around them.

The confirmation of the findings of other authors of changes in the batonnets of the renal cells in stages of secretion is further supported by the demonstration of a lack of secretion in those regenerated cells which do not contain these cell organs. As we have shown, secretion of urea does not occur in the regenerated cells which do not contain the batonnets, and one might therefore infer that secretion depended on these cell organs.

Another interpretation is, however, possible. It may be that the passage of the urinary constituents occurs by some invisible mechanism, and that the passage of these substances merely disturbs the inactive mitochondria which exist there. This process would be disturbed by some undemonstrable fault in the regenerated cells, which do not contain the mitochondrial elements and which could therefore show no change.

Of these two possibilities the first appears to be a more logical and more direct line of reasoning. As the batonnets are the only cell organs we can possibly connect with such a function, as changes are observed in them in periods of active function, and as function is lacking when they do not exist, it does not seem unreasonable to suppose that function is dependent in large part, if not entirely, upon them.[4]

Another point of interest is the relation of the urea granules and the granules of vital dye to the batonnets. Modern investigators all agree that the dye is secreted at least partially by the proximal convoluted tubule, and that there *Speicherung* of the dye also occurs. Aschoff and his pupils (22) claim that the mitochondria are the seat of this storage, though he denies that there is ever a secretion of the granules themselves. He has further shown that excretion of the dye occurs before the granules are stained, and claims that this is due to the secretion of the dye through the glomerulus in the early periods of elimination. Gross (23), on the other hand, admitting the difference in secretion and *Speicherung* time, claims that this can be explained without recourse to glomerular activity. The granules, during secretion of the dye, take it up from the blood and pass it on to the urine. After considerable time they become overloaded with the dye and then accumulation, or *Speicherung*, begins. As we have never, in a large number (128) of vitally stained animals, seen any deposits of dye (carmine and trypan blue) in the glomerulus, we would accept Gross's explanation as the more likely. Though there are certain differences between *Speicherung* and secretion, the former can only occur when the dye is passing through the cells (secretion),

[4] The observations of Cesa-Bianchi (21) on unfixed renal cells in which he observed changes in the granules with variations in the concentration of the surrounding fluid have been offered as explanatory of the changes in the batonnets in stages of diuresis. The inadequacy of such an explanation is apparent when one considers that if such were the case, the mitochondria throughout the entire length of the tubule would be affected along with the variations in the concentration of the urine, and not a single, comparatively short segment (proximal convoluted tubule) alone.

and can therefore be taken as evidence of a secretory process in the renal cells.[5]

The urea granules also appear in definite rows in suitable specimens, and maintain this arrangement even when the contour of the cell is modified by pressure. To explain this constant appearance we can only imagine the urea to be held by some preexisting structures, and there precipitated by the mercuric salts. If we assume the mitochondria, which apparently play a similar part in the secretion and storage of vital dyes, to be the seat of absorption of the urea from the blood stream, the morphological appearances could be easily explained. The absence of the urea granules from cells which do not contain mitochondrial granules is very suggestive of such an explanation. Such an assumption follows the theory of Regaud who claims for the mitochondria the property of selective absorption of substances destined to be secreted from the blood stream, the condensation of these substances in the granules, and the final elimination of them into the lumen of the gland. It is also essentially the idea of the *Condensatoren* advanced by Gurwitz.

Another point which the present investigation emphasizes is the anatomical and functional differences in the proximal and distal convoluted tubule. The unfortunate use of the common term, convoluted tubule, with the prefix of proximal and distal, has led to a misconception in the relation of these two segments to each other. The current conception, and one seen even in standard texts, seems to be that there is little or no difference, anatomically or functionally, in them. As a matter of fact, the distal convoluted tubule is not a convoluted tubule, but merely the cortical part of the ascending limb of the loop of Henle, which at best has but one or two poorly developed kinks in it. This is best seen in isolated tubules (Peter, Huber, Oliver). Differences in the mitochondria (rods shorter) and absence of a cuticular seam further distinguish it from the proximal convoluted tubule. Functionally the differences are even more marked, for, as we have

[5] We cannot assume the dye to be absorbed from the lumen of the tubules, as did Sobieransky and others, as such an explanation has been rendered impossible by the experiments of Gurwitz.

The reader is referred to Policard's monograph for a detailed discussion of the absorption theory.

shown, dye and urea are not secreted by this segment, nor are changes seen in their batonnets during secretion. The adoption of a more accurate terminology would obviate this confusion.[5]

The final point to which we wish to refer is the relation of failure of secretion of urea on the part of the regenerated cells to the increase of non-protein nitrogen of the blood seen in certain forms of kidney disease. Obviously there can be no direct transference of these conclusions to human nephritis, as the chronic uranium nephritis differs entirely from any known human type of kidney disease. Even in the demonstrated "nitrogen retention" in uranium nephritis many obstacles are met in explaining the faulty elimination by so simple a means. As we have shown, a comparatively large amount of tubule apparently secretes urea normally, so that though the failure of the ability of the regenerated cells to functionate may be one factor, many others, and probably more important ones, must be considered, as Mosenthal (24) has shown. It is hoped, however, that these newer methods of morphological investigation may lend them-

[6] The French and German terms, in comparison with the English, are as follows:

French (Policard).	English.	German (Peter).
I. Segment à striation et à cuticle (tube contourné).	Proximal convoluted tubule.	Hauptstück (Konvolut).
Partie corticale.		Pars convoluta.
Partie medullaire.		Pars recta.
II. Segment grêle.	Descending limb of Henle's loop.	Absteigender, dünner Schenkel der H. Schleife.
III. Segment intermédiaire (à striation sans cuticle).		Aufsteigender, dicker Schenkel der H. Schleife.
Partie medullaire.	Ascending limb of Henle's loop.	
		Zwischenstück.
Partie corticale.	Distal convoluted tubule.	Eigentliches Schaltstück.
IV. Segment dit excréteur (sans striation ni cuticle),	Collecting tubule.	Sammelrohr.

selves to combination with functional studies of the kidney, and that by such a combination some light may be cast on the more obscure side of the problems of kidney function, both normal and in disease.

SUMMARY.

1. Definite morphological changes occur in the batonnets of the proximal convoluted tubule in stages of activity.

2. Atypical mitochondria but no batonnets exist in the regenerated cells found in chronic uranium nephritis.

3. No secretion of urea occurs in these cells which do not contain the cell organs.

4. The urea is secreted normally in the proximal convoluted tubule only (Leschke).

5. The urea appears in the form of granules, which are arranged in definite rows.

6. It is suggested that the secretion of the urea is by means of the mitochondrial batonnets which act as condensors.

I take this opportunity to thank Dr. Ophüls for his interest and frequent aid in these investigations, and Drs. Addis and Watanabe for their determinations of the urea content of the urine in several experiments.

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EXPLANATION OF PLATES.

PLATE 49.

FIG. 1. Normal kidney of a guinea pig fixed in Kolster's fluid and stained with iron-hematoxylin to show the batonnets. At the left is seen a glomerulus and around it are the sections of the proximal convoluted tubule which are filled with the long batonnets. Bausch and Lomb obj. 1/6, oc. 1.

FIG. 2. Kidney of a guinea pig on the 15th day of uranium nephritis. Fixation and staining as above. At the upper edge of the figure is seen a glomerulus, and below, six sections of the normal Division I of the proximal convoluted tubule, which contain the batonnets. A normal collecting tubule at A does not contain these structures. The remainder of the tubules are lined with regenerated epithelium, which does not possess any of the batonnets. The large irregular nuclei and indefinite lumen of the regenerated tubule are shown at B. Bausch and Lomb obj. 1/6, oc. 1.

PLATE 50.

FIG. 3. A tubule with regenerated epithelium from Fig. 2. The large irregular nuclei and indefinite lumen are well seen. In the protoplasm of the cells there are many poorly staining granules but no definite batonnets. In the lower left corner is a collecting tubule which normally does not possess the rodlets. Note the difference in size and shape of the normal nuclei in this tubule and those of the regenerated tubule. Bausch and Lomb obj. 1/12, oil immersion, oc. 1.

FIG. 4. Kidney of a guinea pig in the stage of urea secretion at low concentration. Leschke preparation. Two terminal divisions of the proximal convoluted tubule. The cells are covered with a definite cuticular seam. The protoplasm is filled with fine granules arranged in rows and connected by fine threads. The apices of the cells are swollen and beginning vacuolization is seen in them. At the left the cut sections of such cell apices fill the lumen. Bausch and Lomb obj. 1/12, oil immersion, oc. 1.

PLATE 51.

FIG. 5. Low power of kidney of a guinea pig in the stage of secretion of urea at high concentration. The beginning of Division I of the proximal convoluted tubule and the lower sections which lie around the glomerulus are seen. All these tubules are so filled with the urea granule as to obscure all detail, even the nuclei being hidden. The lumina of the tubules contain the poorly fixed cell apices and granular material. Bausch and Lomb obj. 1/6, oc. 1.

FIG. 6. Kidney of a guinea pig on the 15th day of uranium nephritis. The animal was given urea solution and kidney prepared by Leschke's technique. On either side of the section are seen glomeruli and surrounding them the normal Divisions I of the proximal convoluted tubule, which are filled with the dark staining urea. The central part of the figure is filled with the regenerated tubules, which contain practically no urea granules. On close examination the irregular nuclei of the regenerated tubules is evident. Bausch and Lomb obj. 2/3, oc. 1.

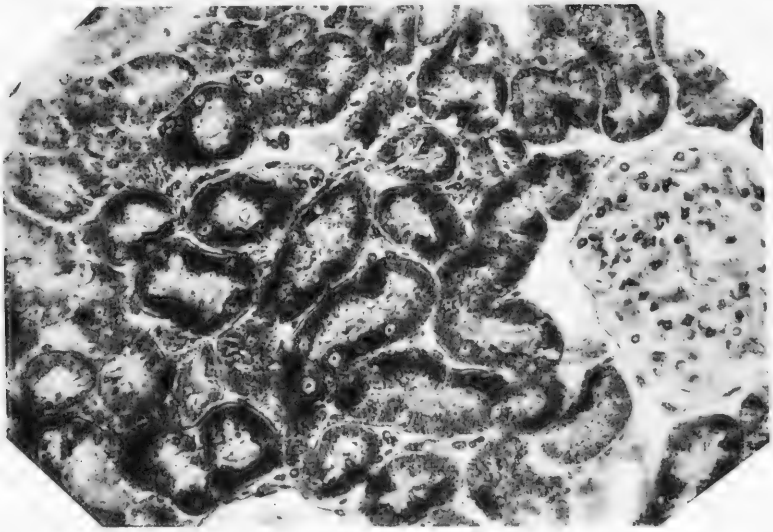

FIG. 1.

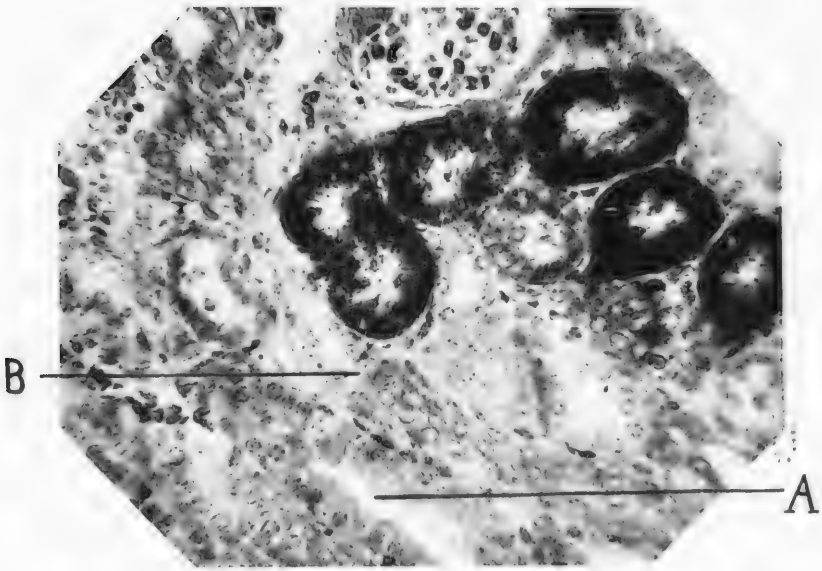

FIG. 2.

(Oliver: Chronic Uranium Nephritis.)

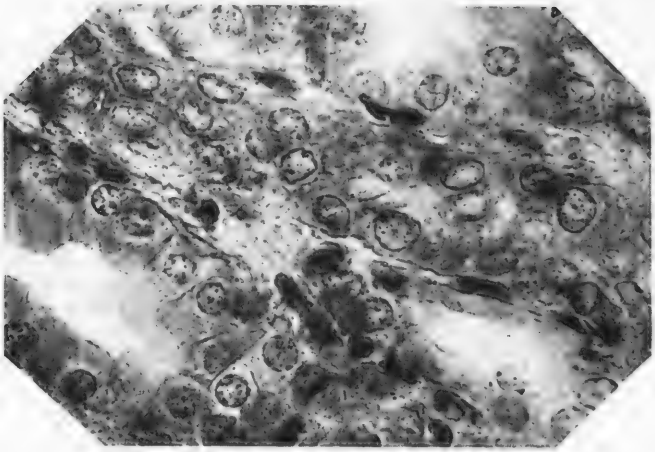

FIG. 3.

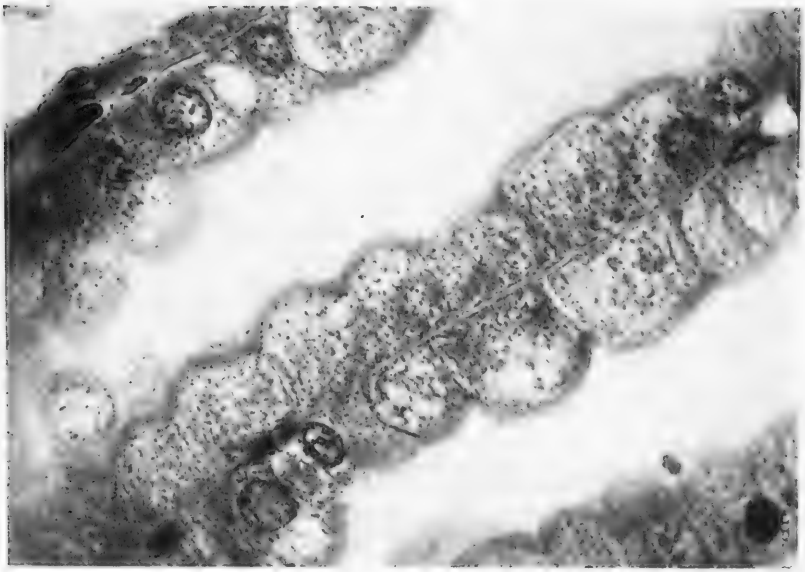

FIG. 4.

(Oliver: Chronic Uranium Nephritis.)

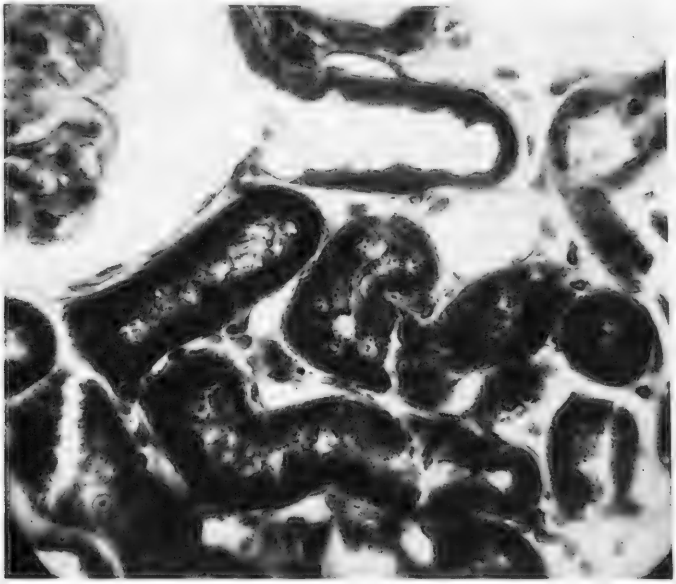

FIG. 5.

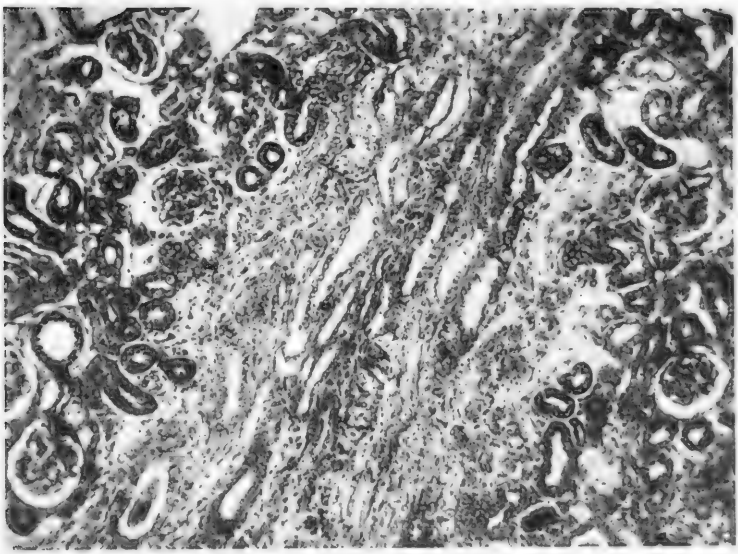

FIG. 6.

(Oliver: Chronic Uranium Nephritis.)

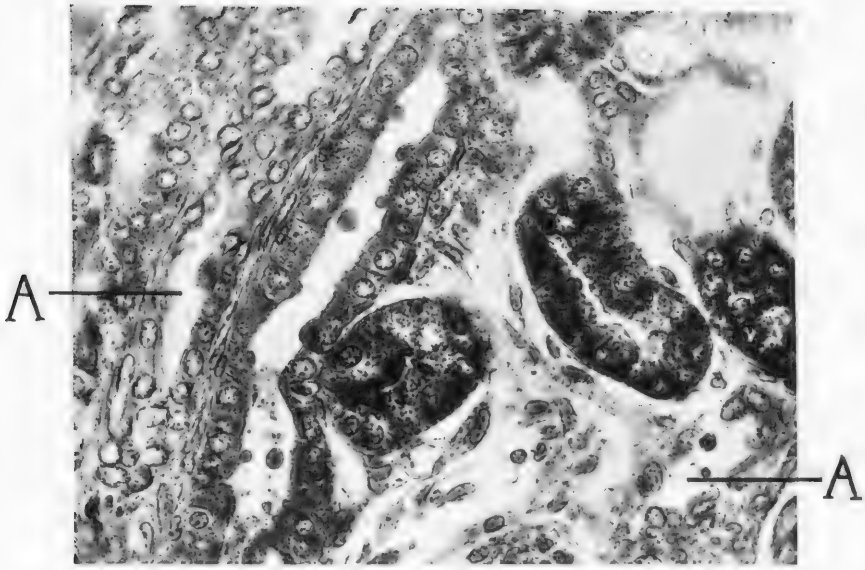

FIG. 7.

(Oliver: Chronic Uranium Nephritis.)

PLATE 52.

FIG. 7. High power of a group of tubules of Fig. 6. Three cross-sections and one longitudinal section of the normal Divisions I of the proximal convoluted tubule are seen filled with varying numbers of urea granules. The connective tissue proliferation around these tubules is evident. The regenerated tubules A are filled with irregularly shaped cells, which are in excess of the normal number, and contain practically none of the urea granules. Bausch and Lomb obj. 1/6, oc. 1.

STUDIES ON TREPONEMA PALLIDUM AND SYPHILIS.

II. Spirochæticidal Antibodies Against Treponema Pallidum.

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(Received for publication, December 30, 1915.)

In a previous communication we have reported on the agglutinins which are formed in rabbits following systematic inoculation with concentrated culture material of *Treponema pallidum*. Incidentally it may be stated that a sheep has also been successfully used. The antigen used for the immunization of these animals was similar to that employed in the first agglutination experiments, except that we have lately used cultures grown on a medium devised by Miss Gilbert in this laboratory containing egg and animal sera instead of tissue and animal sera. The egg cultures which have been used have been made up in a manner similar to that employed in the preparation of Dorset's egg, the egg broth mixture being coagulated in the inspissator, with or without glycerine, in high tubes, the broth serum mixtures poured into the tubes, and these sealed with sterile oil after inoculation. We mention this medium in passing since it promises to furnish an excellent method for obtaining clean antigen, unmixed with tissue detritus, a disadvantage incident to tissue cultures. A subsequent report will be made on this medium. The inoculations in animals have always been made in such a way that cultures containing sheep serum were used for immunization, and the cultures finally used in reaction with the immune serum were grown on ascitic fluid. When tissue cultures were employed, rabbit kidney was used in both cases. The reason for this was that we wished to avoid possible protein-antiprotein reactions which by fixing complement would render our experiments negative.

The spirochæticidal experiments were carried out according to the following general scheme. A series of high tubes, such as are used for treponema cultivation, were prepared, and into them equal quantities of salt solution emulsions or culture fluid emulsions of the treponemata were placed. At first experiments were duplicated with various dilutions of the spirochæte emulsions in order to be sure that we did not use too large a quantity of the microorganisms, an accident which might entirely defeat the possibility of a positive result, in that incomplete killing could not be differentiated from absolute failure to kill. Later we found that emulsions so made up that 10 to 15 spirochætes per dark-field were visible were suitable for the work, and after this, such emulsions in 0.1 cc. quantities, or weaker emulsions in twice this amount, were used throughout.

To the treponemata in these tubes normal serum and immune serum in various dilutions, heated and unheated, and immune serum, inactivated and reactivated with normal serum, were added. Controls with salt solution were always done, and in all cases the experiments were set up in duplicate, sometimes in triplicate sets, in order to obviate the possibility of an occasional negative plant. The total volumes were brought up to 1 cc. in every case, and these tubes were set away in the thermostat at 37.5°C. for 2 to 3 hours. At the end of this time a bit of sterile kidney tissue and ascitic agar were added to each of the tubes, the medium was covered with liquid petrolatum, and the tubes were incubated. Readings were made at the end of from 2 to 3 weeks when the controls had shown active growth.

No experiment was accepted in which the controls were not well developed and in which the duplicates did not check up. Both immune rabbit serum and sheep serum were used in individual experiments.

In Experiment I we submit simply the fact that normal rabbit serum in quantities of 0.1 cc. does not kill the spirochætes, whereas a relatively weak immune serum we had at that time did so, and that spirochæticidal action was also evident where the inactivated immune serum was reactivated with an ineffective dose of normal active serum.

EXPERIMENT I.

Feb. 12, 1915.

Tube No.	Serum.	Amount.	Salt solution.	Spirochæte suspension.	Result.
		cc.	*cc.*	*cc.*	
1 (CIa).	Normal rabbit serum, unheated.	0.1	0.9	0.1	Excellent growth.
2 (CIIa).	Immune rabbit serum, inactive.	0.1			
	Normal rabbit serum, active.	0.1	1.8	0.1	No growth.
3 (CIIIa).	Immune rabbit serum, active.	0.1	0.9	0.1	" "
4 (D).	Salt solution.		1.0	0.1	Growth.

In Experiment II it is apparent that normal serum in quantities of 0.04 cc. no longer exerted spirochæticidal action. Immune serum exerted some spirochæticidal action in this quantity, in that no growth whatever was found in one of the series, and in the other after considerable search a few were found which we judged to be probably remains of those planted, since we know from other experience that spirochætes may remain unchanged for a long time without growing in tubes so prepared, but for the sake of not falling into possible error we listed this as doubtful growth. It is apparent, however, from Tubes DDII that immune serum in quantities of 0.01 cc., reactivated with an amount of normal serum which by itself had no inhibitory action, gave a sterile tube, which therefore indicates the spirochæticidal action in the reactivated serum. The same thing, less strikingly (since there was growth in one of the tubes of the set), is true of Tubes EEII, in which 0.005 cc. of immune inactivated serum was used.

In the preceding experiment sheep serum, normal and immune, was used, and, as agglutination has shown, the sheep had not responded as actively as did rabbits in antibody formation, probably since it had not been easy to get relatively as large amounts into the sheep up to that time as was possible in the case of a rabbit.

In the following experiments normal rabbit and immune rabbit sera were used.

EXPERIMENT II.

Serum from a normal sheep and from a sheep immunized with Culture A were used, according to the following protocol, dilutions being made in salt solution so that the total volume in each tube was 1.0 cc. The inactivated sera were heated at 56°C. for a half hour before dilution. 0.2 cc. of a suspension of spirochætes of Culture A, containing 4 to 6 organisms to the field, was added to each tube and the tubes were incubated for 2 hours at 37.5°C. A piece of sterile kidney tissue and ascitic agar were then added to each tube, the medium was covered with liquid petrolatum, and the tubes were incubated. The experiment was set up in duplicate, and the results on the duplicate tubes are recorded separately.

Oct. 20, 1915.

Tube No.	Serum.	Amount.	Result.	
			Series A.	Series B.
		cc.		
1 (AI).	Normal sheep serum, active.	0.1	Growth.	Growth.
2 (AII).	Immune serum, active.	0.1	No growth.	No growth.
3 (BI).	Normal " "	0.04	Growth.	Growth.
4 (BII).	Immune " "	0.04	" (?)	No growth.
5 (CI).	Normal " "	0.02	"	Growth.
6 (CII).	Immune " "	0.02	"	"
7 (CIII).	Normal " "	0.01	"	"
8 (DDI).	" " inactive.	0.01	"	"
	" " active.	0.02		
9 (DDII).	Immune " inactive.	0.01	No growth.	No growth.
	Normal " active.	0.02		
10 (DDx).	Immune " inactive.	0.01	Growth.	Growth.
	Normal " active.	0.01		
11 (EEI).	" " inactive.	0.005	"	"
	" " active.	0.02		
12 (EEII).	Immune " inactive.	0.005	"	No growth.
	Normal " active.	0.02		
13 (EEx).	Immune " inactive.	0.005	"	Growth.
	Normal " active.	0.01		
14 (F).	Salt solution control.	1.0	"	"

In Experiment III it will be seen that while the spirochæticidal action of the normal serum was eliminated below 0.1 cc., no growth occurred in the tubes containing as little as 0.02 cc. of the immune serum.

EXPERIMENT III.

Immune rabbit serum, No. 1 (Culture A used for immunization), and normal rabbit serum were employed. The culture used was one of the homologous Strain A.

Oct. 25, 1915.

Tube No.	Serum.	Amount.	Result.	
			Series A.	Series B.
		cc.		
1 (AI).	Normal serum, active.	0.1	No growth.	No growth.
2 (AII).	Immune " "	0.1	" "	" "
3 (BI).	Normal " "	0.04	Growth.	Growth.
4 (BII).	Immune " "	0.04	No growth.	No growth.
5 (CI).	Normal " "	0.02	Growth.	.....
6 (CII).	Immune " "	0.02	No growth.	No growth.
7 (F).	Salt solution.		Growth.	Growth.

Experiment IV illustrates the fact that the spirochæticidal action of a rabbit immunized with our Strain A was equally active against one of the Noguchi cultures, known as N_3 in our laboratory. In this experiment again immune serum, active, inhibited in quantities as small as 0.04 cc., whereas normal serum did not prevent growth

EXPERIMENT IV.

Immune Rabbit, No. 1; normal rabbit serum; Culture N_3.

Nov. 17, 1915.

Tube No.	Serum.	Amount.	Result.	
			Series A.	Series B.
		cc.		
1 (AI).	Normal serum, active.	0.1	Growth.	Growth.
2 (AII).	" " inactive.	0.1	"	"
3 (AIII).	Immune " active.	0.1	No growth.	No growth.
4 (AIV).	" " inactive.	0.1	Growth.	Growth.
5 (BI).	Normal " active.	0.04	"	"
6 (BII).	Immune " "	0.04	No growth.	No growth.
7 (BIII).	" " inactive.	0.04	Growth.	Growth.
8 (C).	" " "	0.1		
	Normal " active.	0.02	No growth.	No growth.
9 (E).	" " "	0.02	Growth.	Growth.
10 (F).	Salt solution.		"	"

in quantities of 0.1 cc. Moreover, immune serum, inactivated, in quantities of 0.1 cc. reactivated with 0.02 cc. of normal active serum, resulted in complete absence of growth. The additional Tube 7 (BIII) showed that inactivation of the immune serum prevented its spirochæticidal action, showing that the structure of the spirochæticidal bodies is similar to that of the bactericidal bodies known in the case of other microorganisms.

SUMMARY AND CONCLUSIONS.

We believe that our experiments have shown that the serum of rabbits and sheep immunized with cultures of *Treponema pallidum* acquires spirochæticidal properties for these culture spirochætes.

The normal serum of these animals also possesses spirochæticidal action if used in sufficient quantities, and the action of the immune serum represents probably an increase of the antibodies normally present.

Both normal and immune spirochæticidal properties are destroyed by heating to 56°C.

The spirochæticidal action of the immune serum can be reactivated by the addition of fresh normal serum of the same species, insufficient in amount to exert a spirochæticidal effect by itself.

The structure of these spirochæticidal bodies, therefore, is entirely analogous to that of the well known bactericidal antibodies known to exist in antibacterial sera.

We do not wish to have these results interpreted as applying to virulent spirochætes as well as to culture spirochætes. A subsequent publication will demonstrate why we specify this at present.

STUDIES ON TREPONEMA PALLIDUM AND SYPHILIS.

III. The Individual Fluctuations in Virulence and Comparative Virulence of Treponema Pallidum Strains Passed through Rabbits.

By HANS ZINSSER, M.D., J. G. HOPKINS, M.D., and MALCOLM McBURNEY, M.D.

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(Received for publication, December 30, 1915.)

In the course of the experimental work carried out at this laboratory in connection with antibody formation against *Treponema pallidum*, it has been necessary to keep a number of strains of this microorganism alive, in a virulent condition, by passage through rabbits. Incidental to this work we have felt it desirable to study fluctuations in virulence in individual strains and to determine, if possible, whether there is a variation in virulence between races of treponema obtained from different human sources.

A great deal of work has been done on rabbit syphilis, the most extensive study probably being that of Uhlenhuth and Mulzer.[1] These writers observed a distinct increase of virulence in the course of rabbit passage. They found that after the fourth passage they often had 69 per cent to 70 per cent of takes, which rose to a frequent 100 per cent after the 11th or 12th generation, with a coincident progressive tendency for generalization.

Noguchi,[2] in doing his earlier work on rabbit syphilis, noticed a distinct difference in the morphology of various strains of treponemata in that some strains were conspicuously thinner than others, and that this morphological difference has some direct correlation to ease of cultivation, agility of motion, and infectiousness. He describes three forms: a thin one with an average width of 0.2 of a micromillimeter, a thick form with an average thickness of 0.3 of a micromillimeter, and an intermediate one measuring 0.25 of a micromillimeter. He states that the thicker types do not produce any changes for 5 to 6 weeks and then

[1] Uhlenhuth, P., and Mulzer, P., *Arb. a. d. k. Gsndhtsamte.*, 1913, xliv, 307.

[2] Noguchi, H., *Jour. Exper. Med.*, 1912, xv, 201.

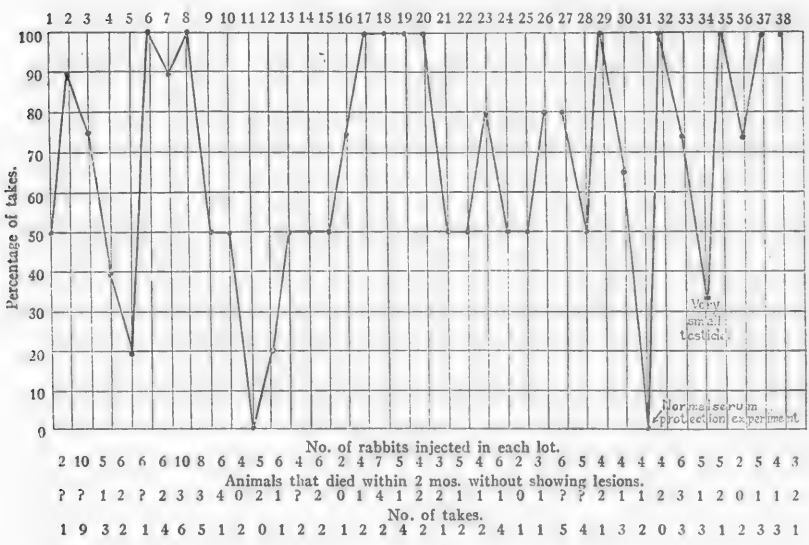
result in hard, indurated nodules which are sharply demarcated, while the thinner ones usually have a shorter incubation time, producing a swelling of the testicle in 10 to 14 days, which by gradual swelling is transformed into a large diffuse lesion. Nichols[3] has studied a strain derived from the central nervous system; that is, from spinal fluid in a case of nervous relapse following salvarsan. It corresponded to the thick form of Noguchi, and Nichols confirms the observation that this type produces hard cartilaginous nodules. He states that the lesions developed by his strain were easily differentiated from the soft, edematous processes produced by the strains obtained from chancres and mucous patches, and calls attention to the fact that patients with nervous relapse often show papular rather than macular secondary eruptions. He states that his strain had a characteristic localization on the scrotal side of the tunica vaginalis and was characterized by a great tendency to generalization, in that it produced chancres on the opposite scrotum, nodules in the opposite testis, keratitis, and nodules on the eyelids, which developed after an unusually short incubation time. It was Nichols' impression that *Treponema pallidum* strains may vary extensively in their invasive powers, and that a certain amount of prognostic deduction can be drawn from a study of the rabbit pathogenicity of a strain isolated from any given case.

These observations are, of course, of great importance for the knowledge of the biology of these microorganisms and might have serious bearing upon the clinical and immunological comprehension of syphilis. We have therefore included a comparative study of the strains that we have used incidental to our work in other fields of observation in the hope of throwing further light upon these relations.

We have inoculated rabbits altogether from fourteen human cases, none of them nervous system cases, about half of them from chancres, and the rest from mucous patches and condylomata. We were successful in starting the strains and carrying them to the second generation in six of these cases. One of these, Strain O, was lost after the third generation; another, Strain K, obtained from the New York Department of Health, was lost after ten generations. The other four have been carried along up to the present day and are now in generations varying from the twelfth to the twenty-first. Strain T, which we have run in addition to these, is Dr. Nichols' strain, which he kindly sent to us in his own seventeenth generation, and which has now gone through an additional nine generations in our hands.

[3] Nichols, H. J., *Jour. Exper. Med.*, 1914, xix, 362.

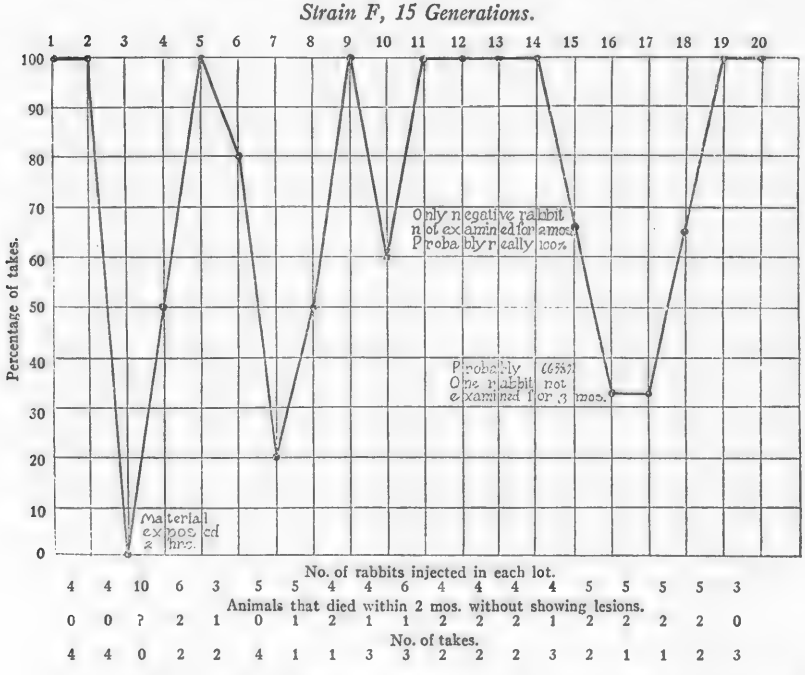
Text-figs. 1 to 5 represent fluctuations in percentage takes of Strains A, T, F, L, and S. The dots on the lines, marking angles, represent not generations, but groups of rabbits inoculated at one time, since often two or three lots of animals were inoculated from various lesions in a single generation. The total number of generations of each strain is given in Arabic numerals in the heading for each chart. The figures at the bottom of the charts represent the number of



TEXT-FIG. 1. Chart showing positive reaction in successive generations of Strain A.

rabbits injected in each lot in the uppermost row, and the number of takes in the lowest row. The middle row represents animals which died within 2 months without showing lesions. Since in the earlier part of our work we were unable to prove out by puncture every lesion, we have omitted from our statistical percentages all doubtful lesions which might have been abscesses and were not proved microscopically, and we have also disregarded in the statistics all rabbits that died within 2 months without showing lesions. It is not improb-

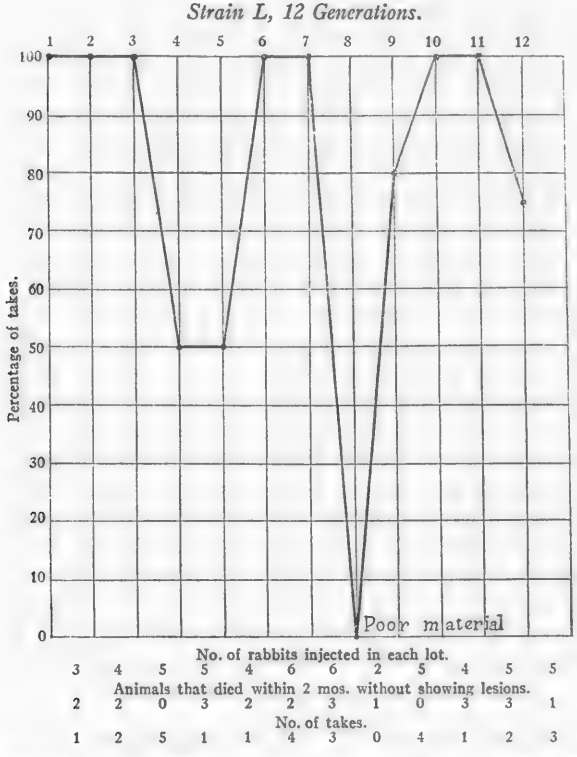
able that *Treponema pallidum* can remain latent for long periods in the testicle without either growing or dying out, and for some reasons, not entirely clear, can later enter into a sudden phase of growth by which a lesion is produced. The work was rendered difficult also (and by this we account for the great irregularities in the charts in certain places) by the fact that in the course of these experiments,


Strain F, 15 Generations.
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
100 90 80 70 60 50 40 30 20 10 0
Percentage of takes.
Only negative rabbit not examined for amos. Probably really 100%
Probably one rabbit examined for 3 mos. 66⅔%
Material exposed 2 hrs.
No. of rabbits injected in each lot.
4 4 10 6 3 5 5 4 4 6 4 4 4 4 5 5 5 5 3
Animals that died within 2 mos. without showing lesions.
0 0 ? 2 1 0 1 2 1 1 2 2 2 1 2 2 2 2 0
No. of takes.
4 4 0 2 2 4 1 1 3 3 2 2 2 3 2 1 1 2 3

TEXT-FIG. 2. Chart showing positive reaction in successive generations of Strain F.

which have covered a period ranging from October, 1913, up to the present time, there occurred occasional epidemics of bacterial infection which killed a large number of rabbits. Often also rabbits of unsuitable size with very small, undeveloped testicles were the only ones that could be procured. Furthermore, on a number of occasions, the material for injection was not the best, and this too tended to make great differences in the number of takes obtained.

When one studies these charts first, without qualifying considerations, in regard to the percentages of takes, the following facts become apparent.

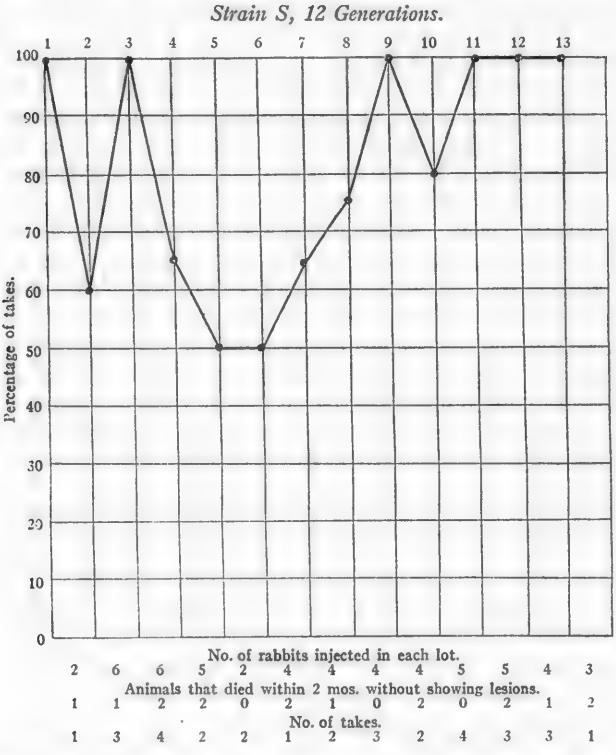

Strain L, 12 Generations.
1 2 3 4 5 6 7 8 9 10 11 12
100
90
80
70
60
50
40
30
20
10
0
Percentage of takes.
Poor material
No. of rabbits injected in each lot.
3 4 5 5 4 6 6 2 5 4 5 5
Animals that died within 2 mos. without showing lesions.
2 2 0 3 2 2 3 1 0 3 3 1
No. of takes.
1 2 5 1 1 4 3 0 4 1 2 3

TEXT-FIG. 3. Chart showing positive reaction in successive generations of Strain L.

Strain A.—One of the rabbits inoculated developed a lesion in the first generation, which, of course, though technically 50 per cent, means nothing considering the small number of rabbits, but in the second generation 90 per cent of the rabbits became syphilitic. In the next generation only 20 per cent, then in quick succession two lots with 100 per cent lesions were found, and after a number of gener-

ations in which in one lot we obtained no takes whatever, again 100 per cent of the rabbits in four successive inoculations were positive.

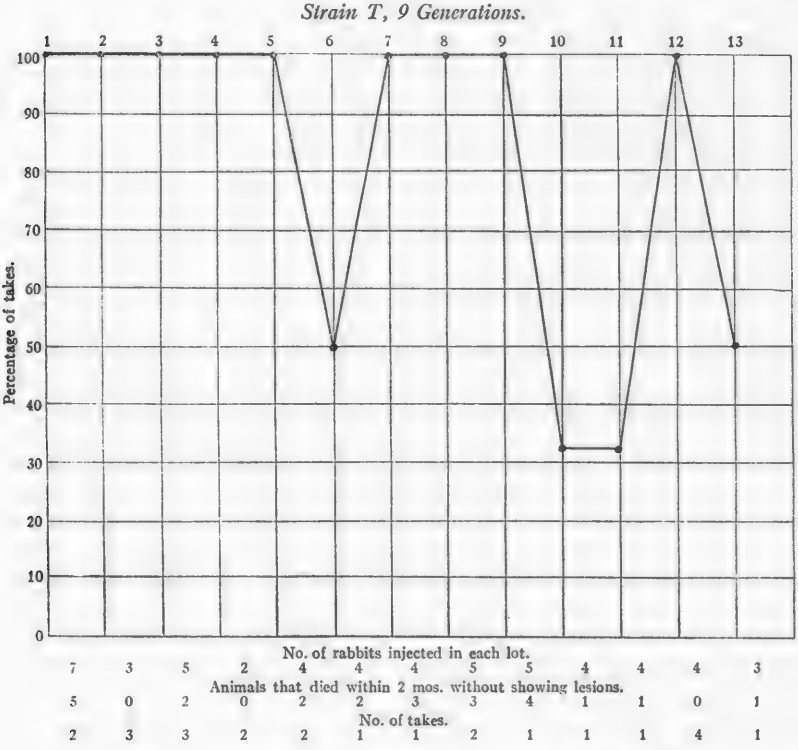
It is not necessary to specify individually the other charts since throughout all of them it is apparent that 100 per cent of takes often occur early in the passage of the strain, which returns again later


Strain S, 12 Generations.
1 2 3 4 5 6 7 8 9 10 11 12 13
100
90
80
70
60
50
40
30
20
10
0
Percentage of takes.
No. of rabbits injected in each lot.
2 6 6 5 2 4 4 4 4 5 5 4 3
Animals that died within 2 mos. without showing lesions.
1 1 2 2 0 2 1 0 2 0 2 1 2
No. of takes.
1 3 4 2 2 1 2 3 2 4 3 3 1

TEXT-FIG. 4. Chart showing positive reaction in successive generations of Strain S.

with fluctuations between, and it is our impression in studying in detail the long series of rabbit inoculations that we have observed, that there is neither a rise nor fall in the virulence of the strains studied by us, but that an originally high virulence possessed by treponema derived from the human body for rabbits, remains high and fluctuates

only according to other factors which we shall discuss later. We do not believe that there is a gradual increase of virulence, as stated by Uhlenhuth and Mulzer, but that these strains remain about the same throughout succeeding generations. Indeed, we believe that with


Strain T, 9 Generations.
1 2 3 4 5 6 7 8 9 10 11 12 13
100
90
80
70
60
50
40
30
20
10
0
Percentage of takes.
No. of rabbits injected in each lot.
7 3 5 2 4 4 4 5 5 4 4 4 3
Animals that died within 2 mos. without showing lesions.
5 0 2 0 2 2 3 3 4 1 1 0 1
No. of takes.
2 3 3 2 2 1 1 2 1 1 1 4 1

TEXT-FIG. 5. Chart showing positive reaction in successive generations of Strain T.

the most favorable conditions for inoculation after the first inoculation from man, when it can be obtained pure and can be injected into suitable rabbits, with favorable technique and in sufficient quantity, 100 per cent of takes, or very nearly that, would be the rule, at least in all the strains studied by us.

The factors upon which we believe that the percentage of takes depends are: (1) The quality of the material. When we have had large diffuse lesions (which are spoken of by most writers as edematous, but in which the soggy, soft mass is filled with a gelatinous, sticky exudate), in which large numbers of treponemata can be obtained in the press juice, which can be injected without much tissue detritus, the chances of obtaining a high percentage of takes are excellent. (2) The type of rabbit injected. We do not believe that the race or color of the rabbit has much to do with its susceptibility to syphilis, since we have seen no difference in this regard. We do believe that intratesticular inoculation is more successful when we have been able to get well grown rabbits with relatively large testicles in which it has been easy to inject directly into the testicular substance and diffuse the material throughout the testicle while slowly pulling out the needle. When small rabbits with undeveloped testicles were used and a good deal of the material has gone into the sack and the other surrounding tissues, the number of takes has been smaller. (3) The technique of injection. We find that the technique of injection has been of great importance. When the material was prepared as described above, the syphilitic testicle macerated thoroughly in a masticator, and often squeezed through cheese-cloth after that to remove tissue particles, and then this relatively clean material carefully injected into the center of the testicle itself, the percentage of takes has been much larger than in cases where the original material consisted of chancres or small indurations, where a reasonable amount of material could be obtained only with tissue detritus.

It seems to us also that some of the irregularity, especially in the early part of our work, may have been due to the fact that we have learned in the course of our studies that great frequency of examination is necessary in order that none of the lesions may be missed. Within the last month we have seen large, diffuse lesions which within 3 or 4 days have diminished in size, changing into small nodules, and in the course of another week have seemed to undergo complete retrogression. Rabbit 2 is an example of this. In this animal a large, diffuse lesion has completely disappeared within the course of a single week. Such rapid retrogression, of course, is noticed only in the cases of large, soft, myxomatous lesions, where there is no hard,

compact infiltration. The nodular and ulcerated lesions do not, of course, manifest the same speed of healing.

Other factors which must be considered in all such statistical studies are the frequent secondary infection or rapid necrosis of lesions and the almost invariable secondary infection of ulcerated lesions, in which even when the original focus was syphilitic, puncture reveals pus and bacteria, and only repeated puncture, and this with the chances against the investigator, may show treponemata under the dark-field.

Statements as to the difference in incubation time possessed by various strains have also engaged our attention, and we have been able to determine no regularity in this regard. In the case of the strains studied by us the incubation times have varied from 14 to over 190 days, and have averaged from 21 to 42 days. In the same strain large fluctuations have appeared, and we believe that this is subject to the modifying factors introduced by the nature of the material and manner of injection on the one hand, and the size and individual resistance of the rabbit injected, on the other. It does not appear from any of our observations that the length of incubation time has any relation at all to special characteristics of an individual strain. The incubation times observed by us were as follows:

Strain A	14	to	48 days.
" F	14	"	71 "
" L	14	"	192 "
" S	8	"	49 "
" T	14	"	74 "

In explanation of the table it should be said that two of the 14 day early lesions were proved by puncture, and that as regards the longest periods tabulated it may well have been that a more frequent examination of the rabbits might have shortened this possibly by as much as 5 days or a week. However, even subtracting such periods, the great length of incubation time in some cases is apparent.

An observation that has been emphasized, especially by Nichols, is the apparent similarity of lesions caused by the same strain, on the basis of which he believes that the human disease may be to a certain extent governed by the type of treponema with which the individual is infected. Although in our earlier studies we did not follow this

point carefully, during the later months of our work we have paid attention to it, and it does not appear that any of our strains, even the one sent to us by Dr. Nichols, has manifested such regularity in pathogenic powers. As illustrative examples we may cite a few only which we think are alone sufficient to show that, in our hands at least, the difference in lesion has been governed by other fortuitous factors rather than by any consistent and inherent properties of the particular treponema used. At the time of writing we have on hand the following lesions:

Strain A.

Rabbit 3. Small nodular lesions in both testicles.
" 4. A hard nodule in one testicle which has followed a large, diffuse lesion in the same testicle.
" 5. A chancre with beginning abscess formation.
" 6. A diffuse, gummy lesion in the left testicle.

Strain F.

" 7. A very small, superficial skin induration and a small nodule in the right testicle. Has had a small nodular lesion in the left.
" 8. A very small central nodule in one testicle.
" 9. A large, diffuse lesion in the right testicle, just beginning to become indurated in the center.

Strain S.

" 10. Two nodules, hard and indurated, one in each testicle at the lower pole. 2 weeks ago this rabbit had had diffuse lesions of the edematous or myxomatous variety.
" 11. Large, diffuse lesions 2 weeks ago which now show as ulcerated nodules or chancres.
" 12. A beginning small, hard nodule in one testicle.
" 13. Soft, general enlargement of the testicle which looks like an early diffuse lesion.

In Strain T, the strain sent us by Dr. Nichols, we have not been able to observe a uniformity of lesions, this strain apparently having changed either because of our technique or by reason of its prolonged rabbit passage. In this strain we have had, it is true, a preponderance of indurated plaques and nodules in the testicle, but in a number of animals, large, diffuse lesions and chancrous lesions have appeared. For instance, in our third and fourth generation of T, we have had the following lesions:

Strain T.

Rabbit 14. Developed a chancre.
" 15. Had an indurated plaque on the left testicle.
" 16. Had a small gelatinous nodule at the globotesticular junction with an edematous or myxomatous appearance of the whole testicle, which was loaded with treponemata. This testicle on palpation appeared to be normal, though generally enlarged.
" 17. Showed large, diffuse lesions of the myxomatous, soft type.
" 18. Another rabbit of the same generation of this strain as Rabbit 17 showed a nodule in the left testicle.

Neither in percentage of takes nor in speed of incubation time has this strain been markedly different from any of our others. The incubation time has varied from 14 to 74 days, which is well within the ranges shown by the others.

Another important point made by previous writers has been studied by us; namely, the relation of difference in morphology to pathogenicity. A distinct difference in thickness, as stated above, has been noted by so authoritative a writer as Noguchi, and we have looked for this in the rabbit *pallida*, often feeling sure that some of the strains did appear more slender and active than others. We have, however, taken lesions of four different strains and examined them with this particular point in view, having three observers check each other on the observation of the slides, without being able definitely to determine consistent difference in size. It is unquestionable that occasionally a strain looks thicker than other strains when taken from the testicle, but we have also seen thick and thin forms together in the same preparation. We have not measured, and do not wish to speak finally of this point, except in so far as we cannot at present definitely confirm consistent differences in morphology between the rabbit strains in our possession. We do not believe that such difference in the morphology, if it exists (and even in Noguchi's hands the difference between the narrowest and the thickest is estimated as 0.1 of a micromillimeter), has a constant relation to difference in invasive property.

CONCLUSIONS.

1. As regards these strains, at least, there is no consistent change, either increase or decrease, of rabbit pathogenicity during progressive rabbit passage.

2. There is no difference in pathogenicity between these different strains, although some of them were isolated from condylomata, some from chancres, one from a mucous patch, and one by Nichols from the nervous system.

3. We do not believe that there is any consistency of difference in speed of incubation between these various strains.

4. We think that the nature of the lesion produced by any individual strain, and the incubation time as well, are dependent upon the fortuitous factors incident to the nature of the material used, the technique of injection, and the size of rabbit in which the injection is made.

As to the tendency to generalize possessed by one strain or another, we are not prepared to make a statement. We have not inspected as carefully as we might all our rabbits in regard to the more obscure syphilitic lesions such as small nodules on the eyebrows, nodules on the nasal bones, and internal lesions of the eye. As a matter of fact, we have noticed three cases of keratitis, probably syphilitic, and a number of preputial metastases, we have seen occasional nodules about the anus, and in one case a peritonitis in the fluid of which living treponemata were found on examination. There has not been in our series an extensive number of generalizations. We attribute this largely to the fact that all the studies here recorded were made on rabbits intratesticularly inoculated. It appears from the writings of other investigators that intravenous and intracardial injection of rabbits leads to a more extensive metastatic or general distribution. Two of the few rabbits inoculated intravenously showed testicular lesions. A series of rabbits intracerebrally inoculated through trephine openings showed nothing definite on autopsy, though one of them showed interference with reflexes and rigidity of the limbs for a time which seemed significant of pathological change. This rabbit, however, recovered entirely.

STUDIES ON TREPONEMA PALLIDUM AND SYPHILIS.

IV. The Difference in Behavior in Immune Serum between Cultivated Non-Virulent Treponema Pallidum and Virulent Treponemata from Lesions.

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I. Agglutination.

In a paper published by two of the writers[1] in June, 1915, it was shown that, as other workers, notably Kolmer,[2] had found before, the inoculation of rabbits with culture *pallida* gives rise to the formation of powerful agglutinating properties in the sera of the animals. Definite agglutination was observed in dilutions of 1: 4,000, and the serum agglutinated not only the strain with which the rabbit had been immunized, but other strains, such as those cultivated by Noguchi. On the specific relationship of these agglutinins to treponemata other than *pallida* we shall report in another paper. The discovery of specific antibodies against the *pallida* in treated animals, of course, again gave us hope that it might be possible to determine whether or not the patient afflicted with active syphilis produced antibodies against the organism, a fact which has been much discussed and written about, but which up to the present time is uncertain. It is not our intention in this communication to review the literature of antibody formation in syphilis, which is extensive though disappointing as to results. We leave this for another paper in which we shall report observations on the presence of agglutinins for culture treponemata in the sera of human beings in the various stages of syphilis.

[1] Zinsser, H., and Hopkins, J. G., *Jour. Exper. Med.*, 1915, xxi, 576.
[2] Kolmer, J. A., *Jour. Exper. Med.*, 1913, xviii, 18.

In this paper we wish to call attention to what seems to be to us a fundamentally important fact; namely, the existence of a definite difference between the treponemata artificially cultivated and those derived directly from the lesions of infected man and animals.

In previous studies carried on in the course of the last two years, we have frequently looked for changes in motility and for agglutination of treponemata found in exudates from syphilitic lesions to which the sera of immunized rabbits had been added. Our results on these occasions were as inconclusive as those of other writers.

Hoffmann and von Prowazek[3] reported in 1906 that the serum of syphilitics in the later stages of the disease produced a diminution of motility in *Treponema pallidum*, an observation which was confirmed by Zabolotny[4] soon afterwards. Contradictory results, however, were obtained by Landsteiner and Mucha, [5, 6] who were unable to observe either immobilization or agglutination in syphilitic serum. Uhlenhuth and Mulzer[7] never observed agglutination in the sera of animals treated with virulent treponemata. Neisser and Bruck,[8] summarizing the work done up to 1911, express themselves as uncertain whether or not agglutinins in the serum of syphilitics had really been proven up to that time, when, of course, work with pure cultures had not been possible. The recent work of Kissmeyer[9] deals with the agglutination of culture treponemata in normal and syphilitic serum and will be left for consideration in a later paper of our own dealing with the same subject.

It is plain, therefore, that in the matter of agglutinin formation in the course of syphilis, nothing is yet definitely known, a state of affairs which unfortunately describes our knowledge of the general subject of antibody formation in this disease.

Having obtained powerful agglutinins in rabbits and sheep treated with culture treponemata, we undertook to determine the action of these sera upon the microorganisms obtained directly from lesions,

[3] Hoffmann, E., Cited from Neisser and Bruck, *Beitr. z. Path. u. Therap. d. Syph.*, Berlin, 1911, 205.

[4] Zabolotny, D., and Maslakowetz, *Centralbl. f. Bakteriol., Ite Abt., Orig.*, 1907, xliv, 532.

[5] Landsteiner, K., and Mucha, V., *Wien. klin. Wchnschr.*, 1906, xix, 1349.

[6] Landsteiner, K., and Mucha, V., *Centralbl. f. Bakteriol., Ite Abt., Ref.*, 1907, xxxix, 540.

[7] Uhlenhuth, P., and Mulzer, P., *Arb. a. d. k. Gsndhtsamte*, 1913, xliv, 307.

[8] Neisser, and Bruck, *Beitr. z. Path. u. Therap. d. Syph.*, Berlin, 1911, 205.

[9] Kissmeyer, A., *Deutsch. med. Wchnschr.*, 1915, xli, 306.

since to one habitually working with treponemata, a great many differences as to morphology, agility of motion, and primarily, of course, of virulence, between the culture organisms and the lesion treponemata are apparent. We therefore carried out a number of experiments, some of which are detailed in this paper, in which the action of serum was tested, in parallel series, upon both the culture organisms and those obtained from lesions.

After a considerable amount of experimentation it seemed to us that material obtained from human lesions, however good, or from rabbit lesions consisting of small nodules and chancres, was unsatisfactory in that the treponemata were so few in number and so intimately mingled with tissue detritus and blood derivatives that washing never resulted in a sufficient yield of microorganisms for satisfactory microscopic work. Incidentally, we may mention that in all agglutination work we have found it inaccurate to rely upon macroscopic results only; our readings had to be made both macroscopically and microscopically. We therefore chose as material only the large, diffuse, gelatinous lesions obtained frequently in rabbit testicles; and we used for most of our experiments such lesions obtained with our own Strain A, which had been cultivated and with which the immune serum had been produced. This gave us a strictly homologous experiment. In addition, we tested this serum on similar lesions obtained with our F and S strains.

In preparing material the testicles were removed and thoroughly macerated with a masticator. The macerated mass was then squeezed through cheese-cloth and, in favorable cases, a thick, gummy liquid was obtained which swarmed with treponemata, actively motile. Many lesions, of course, were worked over in this way without yielding favorable material, but in the experiments finally accepted only material was used in which a great many *pallida* were present relatively free from admixtures of foreign material. To the gummy fluid salt solution was added and the larger particles which had come through the cheese-cloth were thrown down by slow centrifugation. The *pallida* have apparently a low specific gravity and hardly any of the organisms are removed when centrifugation is neither at high speed nor prolonged. The supernatant fluid from this primary centrifugation was removed and allowed to settle in the

refrigerator over night. This removed more of the detritus. Dilution with salt solution was then practiced and very prolonged centrifugation, 3 to 4 hours at high speed, brought down most of the treponemata. These were washed in salt solution, and if considerable admixtures of tissue particles, etc., were still present, repetition of the process with more or less variation according to the nature of the material was practiced. When the final preparation was obtained, it usually consisted of about 0.5 to 1.0 cc. of a salt solution suspension, relatively clean of detritus, containing from four to six treponemata to the field, sometimes less, but in some cases considerably more. With material so prepared the experiments were set up.

EXPERIMENT I.

Agglutination of Virulent Treponemata with Serum of Rabbit 1.

Readings were taken macroscopically and microscopically after 2 hours at 37°C. and after 14 to 16 hours in the refrigerator.

The suspension of the Strain A culture material was made approximately equivalent to the virulent suspension.

Tubes set up.	Agglutination.
Strain A culture + Rabbit 1 serum, 1: 10.	+++
" " " + normal serum, 1: 10.	±
Virulent Strain A + Rabbit 1 serum, 1: 10.	±
" " " + normal serum, 1: 10.	±

EXPERIMENT II.

Comparative Agglutination of Virulent Strain A and Culture Strain A.

Very good, spongy lesion used; treated as described above. A suspension of Culture A was made which corresponded in number of treponemata to the virulent suspension obtained.

Serum of Rabbit 1 was used. This serum agglutinated Culture A in dilutions of 1: 2,000.

Tubes set up.	Agglutination.
1. Virulent Strain A + Rabbit 1 serum, concentrated.	±
2. " " " + " 1 " 1: 20.	−
3. " " " + salt solution.	−
4. Culture " " + Rabbit 1 serum, concentrated.	+++
5. " " " + " 1 " 1: 20.	++
6. " " " + salt solution.	−

EXPERIMENT III.

Strain S.

Suspension of Treponemata from a Gelatinous Lesion in a Rabbit Testicle.

Readings were made macroscopically and microscopically.

The suspension of Strain A culture material was made approximately equivalent to the virulent suspension.

Tubes set up.	Results.		
	1 hr. at 37° C.	2 hrs. at 37° C.	18 hrs. in ice chest.
1. Virulent Strain S + Rabbit 1 serum, 1:4.	0	0	0
2. " " " + normal rabbit serum, 1:4.	0	0	0
3. Culture Strain A + Rabbit 1 serum, 1:4.	+++	+++	+++
4. " " " + normal rabbit serum, 1:4.	±	±	++

EXPERIMENT IV.

Suspension of Virulent Strain S Used.

Readings were taken macroscopically and microscopically after 2 hours at 37°C. and after 14 to 16 hours in the refrigerator.

Tubes set up.	Agglutination.
Strain A culture + Strain A serum, 1:3.	+++
" " " + salt solution.	−
Virulent Strain F + Strain A serum, 1:3.	±
" " " + salt solution.	−

EXPERIMENT V.

Suspensions unfortunately very thin. Therefore Strain A culture suspension was made equally thin, about three or four microorganisms to the field.

The different columns represent readings by three different men in order to be sure that no individual error of judgment was possible.

Tubes set up.	Results.		
1. Virulent treponemata 3 drops + Rabbit 1 serum, 1:5, 3 drops.	−	−	−
2. Virulent treponemata 3 drops + Rabbit 1 serum, 1:20, 3 drops.	−	−	−
3. Virulent treponemata 3 drops + normal serum, 1:5, 3 drops.	−	−	−
4. Culture Strain A 3 drops + Rabbit 1 serum, 1:5, 3 drops.	++	++	++
5. Culture Strain A 3 drops + Rabbit 1 serum, 1:20, 3 drops.	++	++	++
6. Culture Strain A 3 drops + normal serum, 1:5, 3 drops.	+	++	++

These experiments show that an immune serum produced by the inoculation of rabbits with a culture of *Treponema pallidum*, although it contained powerful agglutinating properties for the culture *pallida*, had little or no effect upon the virulent *pallida* obtained directly from lesions. In Experiments II and IV there was slight but definite indication that a very weak effect had been produced upon the virulent treponema by the immune serum. We shall refer to this again, since it seems to indicate that if a sufficiently strong immune serum could eventually be produced, even the virulent *pallida* might be influenced. We need only refer to the long delayed success in passive immunization to the pneumococcus, where the capsulated nature of the organism rendered it unamenable to anything but the most powerful immune serum.

Furthermore, this apparent inability of the virulent *pallida* to go into relation with an antibody so powerfully active against the culture organism, going hand in hand with a loss in virulence on the part of the culture treponema, suggested the possibility of a change physiologically not unlike that associated in some of the bacteria with the acquisition and loss of a capsule.[10] Moreover, the difficulties of staining and general appearance variations of thickness and thinness observed in the *pallida* strains, together with the sticky, gelatinous nature of the lesions, also suggest to the observer the possibility that these organisms may either be capsulated or have a protoplasmic structure chemically not unlike that of the capsules of bacteria.

It occurred to us that we might be able to produce agglutinability in the virulent *pallida* by subjecting them to the acid and heat treatment introduced by Porges in experiments with the Friedländer bacillus group. Accordingly we carried out experiments in which virulent *pallida* material was treated with hydrochloric acid in a total concentration of 0.0625 N and heated in a water bath to from 70° to 80°C. for 15 minutes. The mixtures were made in very small agglutination tubes and measured with capillary pipettes by the Wright method, since, of course, it was difficult to obtain any quantities of the material for examination.

[10] Each of the writers individually has seen in virulent *pallida* suspensions occasional sheath-like structures which resemble capsules and which we have never seen in culture suspensions. We cannot yet stain these and the uncertainty of light effects in dark-field observations causes us to hesitate in making a positive statement.

EXPERIMENT VI.

Virulent Treponemata, Strain F.

Good lesion; treated as in preceding experiments.

Washed thoroughly in order to prevent the disturbing effects of possible coagulation of protein by the acid and heat treatment. Parts of the suspension were then made up to 0.0625 N hydrochloric acid and heated for 15 minutes at 80°C., and the volumes of the other fractions made equal with salt solution.

Tubes set up.	Result.	
1. Virulent Strain F, unheated, + salt (control).	+	Many free treponemata and few clumps of 3 to 8 individuals.
2. Virulent Strain F, unheated, + serum of Rabbit 1, 1:20.	++	Few free treponemata. Clumps larger than control.
3. Virulent Strain F, unheated, + serum of Rabbit 1, 1:50.	+	Perhaps slightly better clumping than control, but uncertain.
4. Virulent Strain F + acid + serum of Rabbit 1, 1:20.	+	Few treponemata caught in clumps of precipitate. Cannot make definite reading.
5. Virulent Strain F + acid + serum of Rabbit 1, 1:50.		Few treponemata caught in clumps of precipitate. Cannot make definite reading.
6. Culture Strain A + serum of Rabbit 1, 1:20.	++	
7. Culture Strain A + serum of Rabbit 1, 1:50.	++++	

EXPERIMENT VII.

Suspension made from material from large, diffuse lesion, Strain A, by the technique described above. Unusually good suspension obtained.

The following tubes were set up:

1. Suspension in 0.0625 N hydrochloric acid.
2. Suspension in salt solution.
3. Suspension in salt solution.

} Heated at 75°C. for 15 minutes.

After two of the tubes had been heated for 15 minutes as above, Immune Serum 1 was added in concentration of 1: 25 to all three of the tubes.

No agglutination was observed in any of them after 48 hours' observation, macroscopically and microscopically.

The acid and heat experiments detailed above (Experiments VI and VII) necessarily consumed much time, and had to be repeated a number of times before we felt sure of the results. We are confident after critical repetition, however, that heating with acid, which according to Porges is sufficient to render inagglutinable capsulated bacteria agglutinable, does not have the same effect upon *Treponema pallidum*.

II. Treponemicidal Action.

In a preceding paper two of the writers have shown that the sera of rabbits and sheep immunized with culture *pallida* possess treponemicidal action upon culture *pallida* in dilutions higher than those possessing similar properties in the case of normal sera of rabbits and sheep. The experiences detailed above which indicated differences between the cultivated microorganisms and the virulent ones obtained from lesions in respect to agglutinability, led us to investigate also the treponema-killing properties of culture immune rabbit serum for virulent *Treponema pallidum*. Accordingly, taking serum from treated rabbits we allowed this serum to act upon treated organisms of varying kinds before injection into normal rabbit testicles. These experiments were as follows (Experiment I).

EXPERIMENT I.

Material from a lesion in Rabbit 19, A XIV. The testicular mass was macerated, the coarser particles thrown down in the centrifuge, and the supernatant fluid, which contained many motile treponemata, was divided into three parts, as follows:

I. 2.5 cc. virulent suspension + 5 cc. immune serum of Rabbit 20 (freshly taken and active).
II. 2.5 cc. virulent suspension + 5 cc. normal rabbit serum.
III. 2.5 cc. virulent suspension + 5 cc. salt solution.

These mixtures were allowed to stand for 1 hour at 37.5°C. There was no loss of motility or clumping in any of them. After 1½ hours it seemed that there were somewhat more non-motile forms in I than in the others. This appears to have been probably a deception. After 2 hours injections were made as follows:

0.75 Cc. Injected into Each Testicle.

I.	Rabbit	21.	Died after 26 days. Negative.
	"	22.	" " 26 " Positive lesion.
	"	23.	Killed for transfers after 40 days. Positive lesion.
	"	24.	Negative.
II.	"	25.	Positive.
	"	26.	"
	"	27.	Negative.
	"	28.	Positive.
III.	"	29.	Died after 26 days. Negative.
	"	30.	Positive.
	"	31.	Negative.
	"	32.	"

In this experiment it is plain that there was no protective power exerted by the action of the serum on the microorganism.

EXPERIMENT II.

Experiment carried out as before, except that no salt solution control was made. The suspension of virulent treponemata was divided into two parts, as follows:

I.	Immune serum of Rabbit 33	5 cc.	+	virulent suspension	2.2 cc.
II.	Normal "	5 "	+	" "	2.2 "

0.8 Cc. Injected into Each Testicle.

I.	Rabbit	34.	Negative.
	"	35.	"
	"	36.	Died before of value.
	"	37.	Positive.
II.	"	38.	Negative.
	"	39.	"
	"	40.	"

Experiment II, also, was entirely negative.

Since we had found in our treponemicidal experiments carried out upon culture organisms that the serum, just as in the case of bactericidal reactions, could be inactivated and reactivated, it seemed to us that insufficiency of alexin or complement might have been a source of error in our previous experiments. Therefore Experiment III was done.

EXPERIMENT III.

Virulent material suspended as before. The material was then divided into three parts and the following mixtures were made:

I.

	cc.
Virulent suspension	1.5
Immune serum, active	3.0
Normal rabbit serum	1.0

II.

Virulent suspension	1.5
Immune serum, active (dilution 1:20)	3.0
Normal serum	1.0

III.

Virulent suspension	1.5
Normal serum, active	4.0

These mixtures were incubated for 2 hours at 37.5°C. At the end of this time rabbits were inoculated in both testicles as follows:

1.0 Cc. Injected into Each Rabbit.

I.	Rabbit	41.	Negative.
	"	42.	"
	"	43.	Positive.
	"	44.	Negative.
II.	"	45.	"
	"	46.	Positive.
	"	47.	Doubtful.
	"	6.	Positive.
III.	"	48.	Negative.
	"	49.	Positive.
	"	50.	"
	"	51.	Negative.

These experiments as far as they have gone show no definite action of the immune serum upon the virulent treponemata, a result which is analogous to the agglutination experiments described in the first part of this paper.

It might be added that the technique by which these experiments had to be done still leaves considerable possibility of error. It has been impossible to obtain the virulent material free from a certain amount of tissue detritus, and dissolved protein which might in some way neutralize the action of antibodies in the immune serum. Fur-

thermore, it is possible that the serum may have definite action and yet the number of treponema placed in the mixtures in each case might have exceeded the maximum quantity that could be taken care of by the amounts of serum used, since, of course, it is well known to one who has carried out *in vitro* bactericidal experiments, that an excessive proportion of microorganisms will result in completely using up the immune serum, leaving unkilled bacteria in sufficient number to cloud by their growth all the bactericidal action. However, this latter possibility is, we think, rendered more or less unlikely by the last experiment where relatively large amounts of serum were used. We consider that these experiments justify the following conclusions, which to us indicate an important principle in questions of immunity in syphilis.

CONCLUSIONS.

Although antibodies can be produced by the immunization of animals with cultivated *Treponema pallidum*, and although these antibodies exert specific agglutinative and treponemicidal action upon the culture organisms, they possess, at least in the concentration so far obtained by us in rabbits and sheep, practically no action for virulent treponemata obtained directly from lesions. There seems to be in the infected body an inability to exert a purely serum action upon the virulent treponemata, a condition of affairs which may well lead to a lack of antigen absorption on the part of the body and a consequent failure to produce serum antibodies.

We do not think that this should in any way discourage our further investigation of the protective action of antibodies produced with culture *pallida*. On the one hand, the slight occasional agglutination and the lower proportion of takes with the concentrated serum in the last experiment at least indicate the possibility that we have been working with sera that are not sufficiently powerful and that just as with work with the pneumococcus and other highly invasive organisms, a serum of considerable antibody contents must be used before results can be expected. Again, the destruction of treponemata and the healing of lesions which undoubtedly takes place in rabbits, sometimes with surprising speed, may be a cellular destruction, and by injecting the sera either locally or intravenously and giving them time

to be absorbed by the cells before injecting virulent material, better results may be obtained. This direction of research as well as further studies on the antagonistic cellular processes against the *pallida*, the immunization of animals with killed virulent organisms, and the antibodies in rabbits and human beings during the course of infection and after recovery are being investigated, and we hope to be able to report upon them in the near future.

AN EXPERIMENTAL STUDY OF PAROTITIS (MUMPS).

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PLATES 53 TO 55.

Studies dealing with the etiology of mumps have been comparatively few in number, and fall into two groups. In the earlier investigations, made chiefly in France,[1] but also in Germany[2] and in America[3] ordinary bacteriological methods were employed, and cocci were isolated from the blood, saliva, and fluid aspirated from the swollen parotid glands.

Attempts to produce parotitis in animals by the inoculation of these cocci proved entirely unsuccessful and the negative results of Roux and Pasteur,[4] and Fichera,[5] who failed to find the cocci demonstrated by other observers, further show that these early studies did not solve the problem of the etiology of epidemic parotitis. The studies made since 1908, when Granata[6] attacked the subject from a new point of view by using the filtrate of patients' saliva for animal inoculation, laid more stress on the reproduction of the disease in laboratory animals than on the isolation of a bacterium from human cases. Granata was the first to conclude that the virus of parotitis may be filterable, basing his conclusions upon the results obtained by inoculating the sterile saliva filtrate from two patients into the

[1] Charrin and Capitan, cited by Védrènes, *Mem. de méd. de chir. et de pharm. mil.*, 1882, xxxviii, 167. Laveran and Catrin, *Compt. rend. Soc. de biol.*, 1893, v, 528. Busquet, *Rev. de méd.*, 1896, xvi, 744. Tessier, P., and Esmein, C., *Compt. rend. Soc. de biol.*, 1906, lviii, pt. 1, 803, 853.

[2] Bien and Michaelis, M., *Verhandl. d. XV Cong. f. inn. Med.*, 1897, xv, 441.

[3] Mecray and Walsh, *Med. Rec.*, 1896, l, 440.

[4] Roux and Pasteur, cited by Védrènes, *Mem. de méd. de chir. et de pharm. mil.*, 1882, xxxviii, 167.

[5] Fichera, G., *Bull. d. r. Accad. di Roma*, 1905. xxxi, 29.

[6] Granata, S., *Med. ital.*, 1908, vi, 647, 672.

blood, parotid gland, and subcutaneous tissue of rabbits. A rise of temperature lasting three days followed the intravenous injections, and swelling of the parotid gland one to two weeks in duration resulted from the other inoculations.

Gordon[7] also used a filtrate of the saliva for the intracerebral inoculation of monkeys. Four of the animals died, having developed meningeal symptoms on the fourth day. At autopsy a lymphocytic meningitis was found with marked degenerative changes in the neurons of the cerebral cortex and anterior horns of the cord. Cultures from the meninges remained sterile. One monkey, inoculated intraperitoneally and intravenously, became ill on the eleventh day and showed swelling of the parotids and stiffness of both jaws, but without nervous symptoms. Recovery was complete. Attempts to transfer the "disease" from one monkey to another with filtrates of the brain and cord from the fatal cases proved entirely unsuccessful. The monkey which survived the longest showed an interstitial parotitis and an interstitial hepatitis, as well. Gordon concludes that mumps is due to a filterable virus of comparatively low virulence.

The work of Nicolle and Conseil[8] is interesting because of a mononuclear leukocytosis detected in one of three monkeys into whose parotid glands they had injected fluid aspirated from the parotid glands of children ill with mumps. After an incubation stage of sixteen to twenty-six days, the animals developed fever lasting two to seven days, and the parotid became swollen in one monkey. No bacteria were demonstrable in the fluid obtained from the human parotid glands.

It will be seen that the experiments of Granata and of Nicolle and Conseil give only suggestive, not entirely successful results so far as the reproduction of mumps in animals is concerned, while Gordon's intracerebral inoculations are chiefly interesting in view of the nervous symptoms sometimes exhibited by patients suffering from epidemic parotitis.

[7] Gordon, M. H., *Reports to the Local Government Board on Public Health and Medical Subjects*, London, 1914, N. S., No. 96.

[8] Nicolle, C., and Conseil, E., *Compt. rend. Acad. d. sc.*, 1913, clvii, 340.

EXPERIMENTAL PART.

Intratesticular inoculation of laboratory animals with infected materials has proved an aid to bacteriological research. The testis acts as an enriching medium, making cultural studies more possible. In addition, the pathological changes caused by the growth of the inoculated virus and the clinical symptoms resulting from such changes can be observed. The fact that orchitis is a frequent complication of mumps in human beings suggested that the animals used in an experimental study of the disease should be inoculated into the testis as well as into the parotid. Cultures from the inoculated organs could then be made according to Noguchi's anaerobic method, while the direct results of the inoculations could be studied. The latter only will be given in this paper.

Cats, rabbits, and monkeys were employed, but as neither rabbits nor monkeys gave promising results, the work was for the time being continued with cats alone.

We are indebted to Dr. Leopold F. Haas and Miss Gillaume, through whose courtesy material for study was obtained from children at the Home for the Friendless; to Dr. Alfred F. Hess and Dr. Sophie Rabinoff, who placed material at the Hebrew Infant Asylum at our disposal; and also to Dr. Matthias Nicoll, Jr., who sent us cases from the Department of Health of the City of New York. The method of obtaining material was uniform. Patients with swollen parotids and symptoms of one to three days' duration were asked to rinse the mouth with sterile salt solution and expectorate into a sterile glass dish. Whenever advisable the parotids were gently massaged at the same time. The secretion thus obtained was filtered through a new Berkefeld candle, and the clear filtrate tested for sterility by aerobic and anaerobic methods. It was found to be uniformly sterile.

The cats were anesthetized with ether, and the skin over the parotid region denuded of hair, then carefully cleansed. With a new, sharp needle, it was not difficult to enter the parotid gland and inject 1 cc. of fluid. Very large, old cats were not favorable because they have a thick layer of fat and dense connective tissue over the gland, making it difficult of access. The fluid is more apt to be injected into

this tissue than into the parotid and the resulting periglandular swelling obscures the results. Well grown young cats proved suitable. The testicles were prepared in the same way as the parotid, and no difficulty was encountered in injecting 1 cc. of fluid.

Before anesthesia the temperature of the cats was recorded, and an actual as well as a differential leukocyte count made. These tests were repeated daily throughout the period of observation.

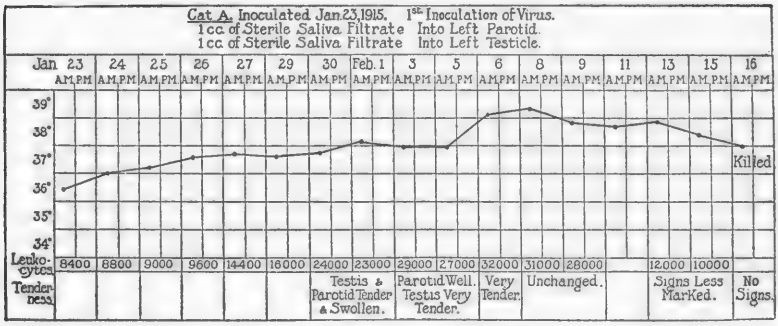
The animals recovered rapidly from the ether and showed no ill effects from the inoculations. The following day the temperature had usually risen 0.5° C., but the white blood cells had not increased in number. There was, as a rule, slight tenderness in the inoculated testicle, and often in the parotid as well. This was evidently of mechanical and not of inflammatory origin, and always disappeared within another twenty-four hours, leaving the cats apparently well on the second day, though the temperature was 0.5 to 0.8° C. above normal. After six or seven days, tenderness returned in the testis, accompanied by swelling; and similar symptoms appeared in the parotid. An increase in the leukocytes became apparent two days after inoculation, and reached the maximum in about seven to fourteen days, coinciding with the height of the fever. The swelling and pain in the parotid lasted two to five days, but the testicular swelling rarely subsided in less than ten to fourteen days. In the third week all the symptoms began to disappear, the leukocytes reaching the normal first, the tenderness disappearing at the same time, and the fever persisting for another week. While tenderness on palpation of the parotids was less marked than that of the testes, and the swelling never reached the stage of marked facial asymmetry, the cats nevertheless manifested some degree of discomfort in the inoculated parotid. The appetite was only slightly affected, and at no period of the experiments did the cats seem especially ill. The disease was not fatal in any instance. The following protocol is typical.

Cat A.—Inoculated Jan. 23, 1915, with sterile filtrate made from the saliva of four children who had been ill two days. 1 cc. injected into left parotid and into left testicle, respectively (Protocol I, Text-fig. 1).

PROTOCOL I.

Date.	Temperature.	Leukocytes.	Remarks.
1915	°C.		
Jan. 23	36.4	8,400	Polynuclears 60; large mononuclears 19; small mononuclears 20; eosinophils 2.
" 24	37.0	8,800	Animal seems well. Left testis slightly tender.
" 25	37.2	9,000	No signs.
" 26	37.6	.....	
" 27	37.7	9,600	No signs. Polynuclears 71; large mononuclears 14; small mononuclears 14; eosinophils 1.
" 29	37.6	14,400	No signs. Polynuclears 73; large mononuclears 14; small mononuclears 13; eosinophils 3.
" 30	37.8	24,000	Left testis quite tender and slightly swollen. Parotid tender. Polynuclears 70; large mononuclears 14; small mononuclears 13; eosinophils 1.
Feb. 1	38.2	23,000	Polynuclears 75; large mononuclears 8; small mononuclears 16; eosinophils 1. Signs unchanged.
" 3	38.0	29,000	Polynuclears 64; large mononuclears 16; small mononuclears 19; eosinophils 1. Left testis very sensitive and swollen. Parotid signs have disappeared.
" 5	38.0	27,000	Signs the same.
" 6	39.2	32,000	Polynuclears 64; large mononuclears 13; small mononuclears 22; eosinophils 1. Tenderness very marked.
" 8	39.4	31,000	No change.
" 9	38.8	28,000	" "
" 11	38.7	.....	
" 13	38.9	12,000	Tenderness less marked.
" 15	38.5	10,000	No tenderness. Left testis slightly smaller than right.
" 16	38.0	.....	Chloroformed.

Left testis smaller; on section it is grey and cloudy, but scarcely firmer than the right; neither hemorrhages nor other lesions apparent. Left parotid no larger than right; on section nothing obvious. Microscopically foci of cellular infiltration are found in both parotid and testis, and epithelial degeneration in the latter. The uninoculated glands on the right side are quite normal.


Cat A. Inoculated Jan 23, 1915. 1st Inoculation of Virus.
1 cc. of Sterile Saliva Filtrate Into Left Parotid.
1 cc. of Sterile Saliva Filtrate Into Left Testicle.
Jan 23 24 25 26 27 29 30 Feb. 1 3 5 6 8 9 11 13 15 16
AM. PM.
39°
38°
37°
36°
35°
34°
Killed
Leukocytes
8400 8800 9000 9600 14400 16000 24000 23000 29000 27000 32000 31000 28000 12,000 10000
Tenderness
Testis & Parotid Tender & Swollen.
Parotid Well. Testis Very Tender.
Very Tender.
Unchanged.
Signs Less Marked.
No Signs.

TEXT-FIG. 1.

Transmission from Cat to Cat.

At intervals varying from seven days to four weeks, the animals were etherized, the inoculated glands removed with aseptic precautions, and extracts or emulsions from them injected into other cats. The extracts were prepared in the following way: The organs were cut into small pieces, thoroughly ground up in a mortar with sterile sand and salt solution in a proportion of about 1 to 10, shaken for 2 to 3 hours at 37° C., and centrifugalized. The opalescent fluid was used for injection after it had been tested for sterility. Emulsions were made by grinding the organs in a small tissue-grinding machine through whose fine wire screen 0.2 to 0.3 cc. of finely divided material passed. This was suspended in 1 to 2 cc. of salt solution and its sterility tested before use. The best time for the reinoculation was found to be from the fourteenth to the seventeenth day when the testicular swelling, the leukocytosis, and the fever were highest. During the first seven to ten days transfers were less uniformly successful. Evidently the reaction requires about two weeks for its maximum development. Atrophy of the inoculated testicle occurred in several cats, after the acute symptoms had abated.

After the third or fourth transfers the reaction developed more rapidly and the effects were more severe. But after the sixth or seventh transfers the reaction perceptibly declined. In one instance only were effects observed at the eighth transfer.

Certain illustrative experiments are given in the text-figures and protocols which follow.

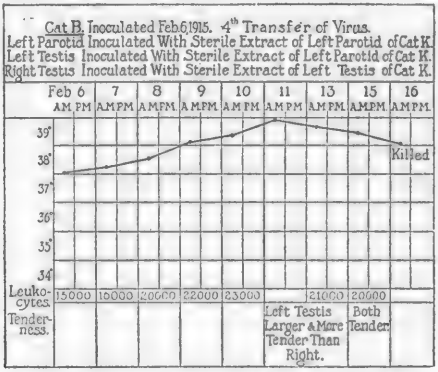
Cat B.—Fourth transfer of Strain III. Inoculated Feb. 6, 1915, with sterile extracts of parotid and testis from Cat K (Protocol II, Text-fig. 2).

Left parotid injected with sterile extract from left parotid.
" testis " " " " " " " "
Right " " " " " " " " testis.

PROTOCOL II.

Date.	Temperature.	Leukocytes.	Remarks.
1915	°C.		
Feb. 6	38.0	15,000	Polynuclears 62; large mononuclears 17; small mononuclears 19; eosinophils 2. No signs.
" 7	38.3	16,000	
" 8	38.5	20,000	Polynuclears 70; large mononuclears 16; small mononuclears 16; eosinophils 4. No signs.
" 9	39.1	.....	
" 10	39.8	22,000	
" 11	39.9	23,000	Polynuclears 76; large mononuclears 11; small mononuclears 11; eosinophils 2. Left testis tender; right less so.
" 13	39.7	21,000	Polynuclears 75; large mononuclears 13; small mononuclears 8; eosinophils 4. Left testis very tender and enlarged.
" 15	39.5	20,000	
" 16	39.0	.....	Chloroformed.

Left testis larger than right; on section both are cloudy and moist compared with those from a normal cat. The left parotid measures 3.5 × 2.5 cm., while the right is 3 × 2.5 cm. in diameter. The left is pinkish in color and more moist and granular on section. Microscopically the right testis shows a more marked degree of "spermatorrhexis" than the left.


Cat B. Inoculated Feb 6 1915. 4th Transfer of Virus.
Left Parotid Inoculated With Sterile Extract of Left Parotid of Cat K.
Left Testis Inoculated With Sterile Extract of Left Parotid of Cat K.
Right Testis Inoculated With Sterile Extract of Left Testis of Cat K.
Feb 6 7 8 9 10 11 13 15 16
AM PM
39°
38°
37°
36°
35°
34°
Killed
Leukocytes
15000 16000 20000 22000 23000 21000 20000
Tenderness
Left Testis Larger & More Tender Than Right.
Both Tender

TEXT-FIG. 2.

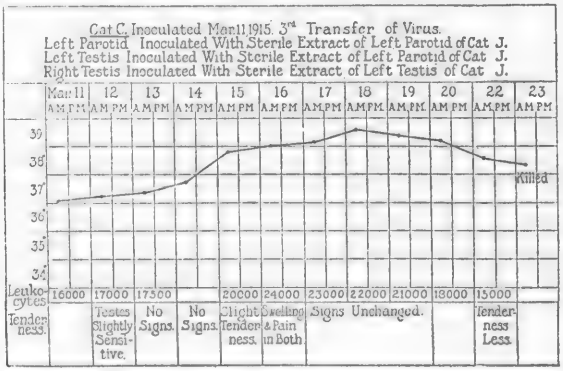
Cat C.—Third transfer of Strain IV. Inoculated Mar. 11, 1915, with sterile extracts of parotid and testis from Cat J (Protocol III, Text-fig. 3).

Left parotid injected with extract from left parotid.
" testis " " " " " "
Right " " " " " " testis.

PROTOCOL III.

Date.	Temperature.	Leukocytes.	Remarks.
1915	°C.		
Mar. 11	37.0	16,000	Polynuclears 62; large mononuclears 16, small mononuclears 18; eosinophils 4.
" 12	37.3	17,000	Testes slightly sensitive.
" 13	37.4	17,500	No signs.
" 14	37.8	.....	" "
" 15	38.8	20,000	Very slight tenderness.
" 16	39.0	24,000	Polynuclears 77; large mononuclears 11; small mononuclears 9; eosinophils 3. Swelling and tenderness in both testes.
" 17	39.2	23,000	Signs the same. Parotid not sensitive.
" 18	39.6	22,000	Polynuclears 79; large mononuclears 6; small mononuclears 10; eosinophils 5. Signs unchanged.
" 19	39.4	21,000	
" 20	39.2	18,000	Polynuclears 70; large mononuclears 4; small mononuclears 18; eosinophils 8. Signs unchanged.
" 22	38.8	15,000	Tenderness less
" 23	38.6	.....	Chloroformed.

Testes scarcely enlarged, but left one, on section, is firmer than the right; both are cloudy. Left parotid is pinkish in color but not larger than the right. Microscopically both testes show marked epithelial degeneration, and, in addition, the left is the seat of cellular infiltration (Fig. 3). Cultures sterile.


Cat C. Inoculated Mar 11 1915. 3rd Transfer of Virus.
Left Parotid Inoculated With Sterile Extract of Left Parotid of Cat J.
Left Testis Inoculated With Sterile Extract of Left Parotid of Cat J.
Right Testis Inoculated With Sterile Extract of Left Testis of Cat J.
Mar 11 12 13 14 15 16 17 18 19 20 22 23
AM PM
39
38
37
36
35
34
Killed
Leukocytes
16000 17000 17500 20000 24000 23000 22000 21000 18000 15000
Tenderness
Testes Slightly Sensitive.
No Signs.
No Signs.
Slight Tenderness.
Swelling & Pain in Both
Signs Unchanged.
Tenderness Less.

TEXT-FIG. 3.

Control Experiments with Normal Organs.

As control experiments, extracts were prepared from the parotid glands and testicles of normal cats, and injected into three healthy cats in doses equal to those previously used. Neither enlargement nor tenderness developed in the glands inoculated in this way nor did the rise in temperature last longer than two days. Coincident with the temperature rise there was a polynuclear leukocytosis of two days' duration. At autopsy no macroscopic or microscopic changes were demonstrable in either the parotids or the testicles, while active spermatogenesis was in progress.

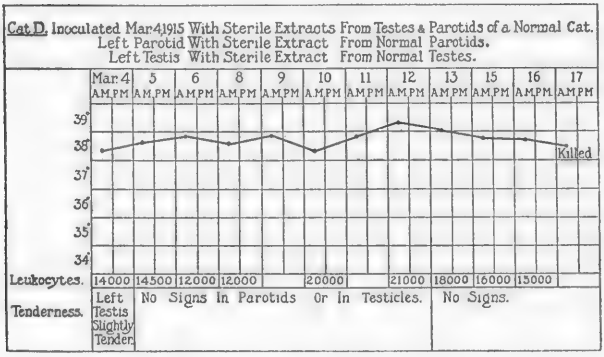
Cat D.—Inoculated Mar. 4, 1915, with sterile extract of parotid and testis from a normal cat (Protocol IV, Text-fig. 4).

Left parotid injected with sterile extract from left parotid.
" testis " " " " " " testis.

PROTOCOL IV.

Date.	Temperature.	Leukocytes.	Remarks.
1915	°C.		
Mar. 4	38.3	14,000	Polynuclears 65; large mononuclears 12; small mononuclears 20; eosinophils 3.
" 5	38.6	14,500	Left testis slightly tender.
" 6	38.8	.....	No signs.
" 8	38.6	12,000	Polynuclears 69; large mononuclears 13; small mononuclears 7; eosinophils 1. No signs.
" 9	38.8	.....	
" 10	38.4	20,000	Polynuclears 65; large mononuclears 10; small mononuclears 12; eosinophils 3. No signs.
" 11	38.9	.....	
" 12	39.4	21,000	Polynuclears 84; large mononuclears 8; small mononuclears 4; eosinophils 4. No signs.
" 13	39.0	18,000	Polynuclears 71; large mononuclears 20; small mononuclears 6; eosinophils 3. No signs.
" 15	38.8	16,000	Polynuclears 64; large mononuclears 28; small mononuclears 6; eosinophils 2. No signs.
" 16	38.8	15,000	Polynuclears 66; large mononuclears 14; small mononuclears 18; eosinophils 2. No signs.
" 17	38.5	.....	Chloroformed.

No difference observed in the parotids and testes on the two sides, on macroscopic and on microscopic examination. Spermatogenesis appeared normal.


Cat D. Inoculated Mar 4 1915 With Sterile Extracts From Testes & Parotids of a Normal Cat.
Left Parotid With Sterile Extract From Normal Parotids.
Left Testis With Sterile Extract From Normal Testes.
Mar 4 5 6 8 9 10 11 12 13 15 16 17
AM PM AM PM AM PM AM PM AM PM AM PM AM PM AM PM AM PM AM PM AM PM
39°
38°
37°
36°
35°
34°
Killed
Leukocytes.
14000 14500 12000 12000 20000 21000 18000 16000 15000
Tenderness.
Left Testis Slightly Tender.
No Signs in Parotids Or in Testicles.
No Signs.

TEXT-FIG. 4

Control Experiments with Normal Saliva.

Other control experiments were made by injecting the sterile filtrates of normal saliva obtained from three healthy persons into the parotid glands and testes of normal cats. The resulting rise in temperature was not greater than 0.5° C. While the leukocytes were only increased by two or three thousand cells, the polymorphonuclear cells were relatively more numerous after the injection, and they did not regain the normal level during the period of observation which lasted from twelve to sixteen days. The small mononuclear cells were relatively diminished. Neither swelling nor tenderness developed in the inoculated glands, and even after the third and fourth successive transfer with this material no macroscopic lesions were discernable at autopsy. On microscopical examination the parotid glands showed no difference between the inoculated and the uninoculated side, and the same was true of the testes in seven of the animals. In two cats, however, the inoculated testis showed a moderate amount of degenerative change in the tubular epithelium. The lesion, however, was not comparable to that in the testes of the cats inoculated with saliva from mumps patients, being far less marked in extent and in degree. It should simply be mentioned that in two instances, the testes of cats into which saliva filtrates from healthy persons had been

injected were not absolutely normal on microscopic examination after death. Protocol V is typical.

Cat E.—Inoculated Oct. 8 with sterile filtrate of normal saliva into left testis and right parotid gland (Protocol V).

PROTOCOL V.

Date.	Temperature.	Leukocytes.	Remarks.
1915	°C.		
Oct. 8	38.6	30,000	Polynuclears 50; large mononuclears 8; small mononuclears 35; eosinophils 7. Slight tenderness of left testis.
" 9	38.3	32,000	Polynuclears 67; large mononuclears 8; small mononuclears 15; eosinophils 10. No tenderness of testis or parotid.
" 11	38.7	33,000	Polynuclears 61; large mononuclears 7; small mononuclears 27; eosinophils 5.
" 13	39.0	29,000	Polynuclears 63; large mononuclears 7; small mononuclears 18; eosinophils 12.
" 14	38.8	32,000	Polynuclears 73; large mononuclears 3; small mononuclears 20; eosinophils 4.
" 15	39.0	30,000	Polynuclears 21; large mononuclears 5; small mononuclears 18; eosinophils 6.
" 16	38.8	25,000	Polynuclears 66; large mononuclears 4; small mononuclears 20; eosinophils 10.
" 18	38.5	22,000	Polynuclears 76; large mononuclears 5; small mononuclears 10; eosinophils 9.
" 19	39.0	32,000	Polynuclears 77; large mononuclears 4; small mononuclears 13; eosinophils 6.
" 20	39.0	30,000	Polynuclears 65; large mononuclears 10; small mononuclears 22; eosinophils 3. No tenderness.
" 21	38.2	.....	Chloroformed.

At autopsy there was no difference in the size, color, or consistency of testes or parotid glands.

Microscopically no lesion was found in the inoculated testis or parotid.

Finally sterile salt solution alone was injected into the parotid gland and testes of three cats. The temperature rose only half a degree, and neither microscopic nor macroscopic lesions developed.

Pathology.

1. Gross Appearance. (*a*) *Parotid Gland.*—On exposing the parotid glands in the recently killed cats, the deeper pink color of the inoculated gland was very striking, as was also its larger size. The difference in weight varied from 70 to 340 mg. The inoculated gland was more moist than the uninoculated, and showed on section a granular appearance, due to swelling of the acini. No other changes occurred. The adjacent lymph nodes were usually congested but not distinctly enlarged (Fig. 5). The molar gland, situated just beneath the skin at the angle of the mouth and extending along the lower lip, was uniformly congested and swollen on the inoculated side. During life the buccal mucosa over the gland was distinctly reddened. This symptom could be found in the cat's mouth in four or five days after inoculation and in a few instances was accompanied by swelling. The opening of the duct leading from the injected parotid was always surrounded by a small zone of congestion.

(*b*) *Testis.*—The inoculated testis was larger than the uninoculated, but unchanged in color. On section the cut surface was more gray, cloudy, and moist. In a few instances the point of inoculation was visible as a small dark spot, but otherwise no focal changes were noted.

2. Microscopic Appearance. (*a*) *Parotid.*—The histological changes when present in the parotid were not constant for all the transfers of every strain. In the first transfer they were usually inconspicuous; and at most congestion of the vessels and swelling of the epithelium of the acini were made out, with edema of the interlobular connective tissue.

The most marked changes appeared coincidently with the third and fourth transfers. In some examples, the glands showed infiltration of the interlobular connective tissue with mononuclear and a few polynuclear cells in addition to the edema The infiltration was most intense about the secretory ducts (Fig. 1) which were sometimes dilated (Fig. 2). The epithelium of the acini was swollen and cloudy in these instances. The areas of cellular infiltration were more pronounced in some parts of the gland than in others, were always multiple, and could be easily differentiated from the small

lymph nodes which are normally present between the lobules. In some instances infiltration did not appear at all.

(*b*) *Testis.*—The histological changes in the testis were more constant than were those in the parotid, and, in further contrast, they were more pronounced in the epithelium than in the supporting framework of the gland. The layer of cells next to the basement membrane of the tubules tended to be normal in appearance, but the rest of the cells were often the seat of a change resulting in a diminution in the number of spermatocytes and consequently of the mitotic nuclei normally found. The spermatids were even more altered, showing as the remains of their nuclei irregular and deeply staining granules, while the cell bodies stained poorly or had undergone extensive lysis. In these cases, spermatozoa, as would be expected, were diminished in number and few were normal in appearance, their broken-up condition being easily discernible (Fig. 3). This process of imperfect spermatogenesis and of disintegration or spermatorrhexis was further indicated by the empty condition of the tubules of the rete and epididymis. Epithelial changes of some degree were present in some part of practically all the inoculated testicles. Interstitial cellular infiltration, on the other hand, occurred far less frequently in the testicle than in the parotid gland. In a few instances it was pronounced about groups of tubules, whose epithelium showed marked degeneration (Fig. 4). Another striking change in the testis was noted in the interstitial cells. These were larger than normal, and, in several instances, actually increased in number (Fig. 3), forming large masses between the tubules.

Fibrous connective tissue in increased amount with a diminution in the size and number of the convoluted tubules was found in the atrophic testes.

The microscopic examinations were always made with material removed from animals killed with chloroform and fixed at once in Zenker's fluid.

Reinoculation Experiments.

Four cats which reacted well were allowed to survive until all symptoms abated and were then reinjected with filtrates prepared from fresh cases of mumps in the manner already described (page

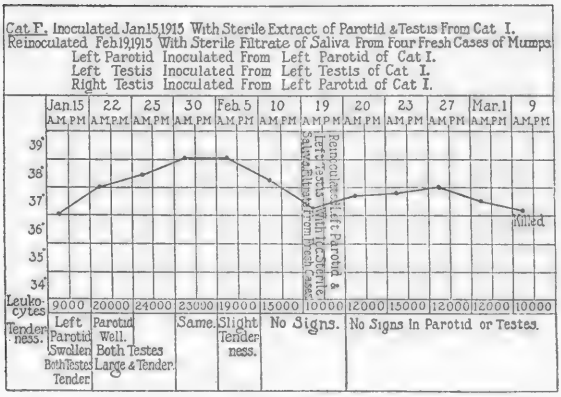
358). As a result of the second injection the temperature rose only 0.8° C., and the leukocytes increased only by 5,000 cells, whereas after the first injection the rise of temperature was about 1.9° C. above normal, and the white cells had more than doubled. No sensitiveness of either parotid or testis developed after the second injection. This result suggested that the first injection had reduced considerably the reaction to the second. Whether the effect was one of immunization can only be conjectured.

Cat F.—Inoculated Jan. 15, 1915, with sterile extract of parotid and testis from Cat I, making this cat the third transfer of this series (Protocol VI, Text-fig. 5).

Left parotid injected with left parotid of Cat I.
" testis " " " testis " " "
Right " " " " parotid " " "

PROTOCOL VI.

Date.	Temperature.	Leukocytes.	Remarks.
1915	°C.		
Jan. 15	37.0	9,000	Polynuclears 66; large mononuclears 17; small mononuclears 12; eosinophils 5.
" 22	38.0	20,000	Polynuclears 80; large mononuclears 15; small mononuclears 5; eosinophils 0. Left parotid tender and swollen; both testes tender.
" 25	38.4	24,000	Parotid swelling gone. Both testes tender and enlarged.
" 30	39.0	23,000	Signs unchanged.
Feb. 5	39.0	19,000	Tenderness of testes slight. Polynuclears 60; large mononuclears 14; small mononuclears 26; eosinophils 0.
" 10	38.3	15,000	No signs. Polynuclears 62; large mononuclears 15; small mononuclears 20; eosinophils 3.
" 19	37.2	10,000	No signs. Reinoculated into left testis and left parotid with 1 cc. of sterile filtrate from 4 fresh cases of mumps.
" 20	37.6	12,000	Polynuclears 68; large mononuclears 15; small mononuclears 15; eosinophils 2. No signs.
" 23	37.8	15,000	Polynuclears 72; large mononuclears 12; small mononuclears 13; eosinophils 3.
" 27	38.0	12,000	Polynuclears 70; large mononuclears 13; small mononuclears 15; eosinophils 2. No signs.
Mar. 1	37.5	12,000	No signs.
" 9	37.2	10,000	" " Chloroformed.


Cat F. Inoculated Jan 15 1915 With Sterile Extract of Parotid & Testis From Cat I.
Reinoculated Feb 19 1915 With Sterile Filtrate of Saliva From Four Fresh Cases of Mumps
Left Parotid Inoculated From Left Parotid of Cat I.
Left Testis Inoculated From Left Testis of Cat I.
Right Testis Inoculated From Left Parotid of Cat I.
Jan. 15 22 25 30 Feb. 5 10 19 20 23 27 Mar. 1 9
A.M. P.M.
39°
38°
37°
36°
35°
34°
Reinoculated Left Parotid & Left Testis With Sterile Saliva Filtrate From Fresh Cases
Killed
Leukocytes
9000 20000 24000 23000 19000 15000 10000 12000 15000 12000 12000 10000
Tenderness.
Left Parotid Swollen Both Testes Tender
Parotid Well. Both Testes Large & Tender.
Same.
Slight Tenderness.
No Signs.
No Signs in Parotid or Testes.

TEXT-FIG. 5.

Serum Protection Experiments.

In order to test the protective power of the serum of a cat which had entirely recovered from an inoculation with the filtrate three months before, three experiments were made. Material from the enlarged glands of cats killed at the height of the reaction had been kept in glycerine in the ice box for four months. This was emulsified in the usual way and the emulsion was left in contact with the serum for two hours at a temperature of 37° C. The mixture was then injected into the parotid gland and testicle of normal cats, control animals being inoculated at the same time with glycerinated material which had not been treated with the serum. A definite difference in the effects was noted in the two sets of animals. The animals receiving the emulsion-serum mixture showed less reaction than those receiving the emulsion alone. Glycerination of the testicles and parotids seemed to make little change, within the period of the experiment, in the results of the inoculation.

The protocols and text-figures illustrating the experiments follow.

1. Control.

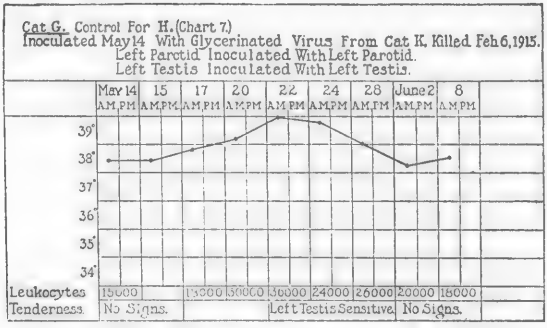
Cat G.—Inoculated May 14, 1915, with glycerinated material from Cat K, killed Feb. 6 (Protocol VII, Text-fig. 6).

Left parotid inoculated with left parotid of Cat K.
" testis " " " testis " " "

PROTOCOL VII.

Date.	Temperature.	Leukocytes.	Remarks.
1915	°C.		
May 14	38.4	15,000	Polynuclears 68; large mononuclears 10; small mononuclears 14; eosinophils 8.
" 15	38.4	.....	No signs.
" 17	38.8	18,000	" " Polynuclears 78; large mononuclears 7; small mononuclears 12; eosinophils 3.
" 20	39.1	30,000	No signs. Polynuclears 70; large mononuclears 13; small mononuclears 8; eosinophils 9.
" 22	40.0	36,000	Left testis sensitive.
" 24	39.8	24,000	" " " Polynuclears 59; large mononuclears 14; small mononuclears 20; eosinophils 7.
" 28	39.0	26,000	Left testis sensitive. Polynuclears 65; large mononuclears 12; small mononuclears 17; eosinophils 6.
June 2	38.3	20,000	No signs. Polynuclears 67; large mononuclears 9; small mononuclears 21; eosinophils 3.
" 8	38.5	18,000	No signs.

Animal survived.


Cat G. Control For H. (Chart 7.)
Inoculated May 14 With Glycerinated Virus From Cat K. Killed Feb. 6, 1915.
Left Parotid Inoculated With Left Parotid.
Left Testis Inoculated With Left Testis.
May 14 15 17 20 22 24 28 June 2 8
A.M. P.M.
39°
38°
37°
36°
35°
34°
Leukocytes 15000 13000 30000 36000 24000 26000 20000 18000
Tenderness No Signs. Left Testis Sensitive. No Signs.

TEXT-FIG. 6.

2. Serum Experiment.

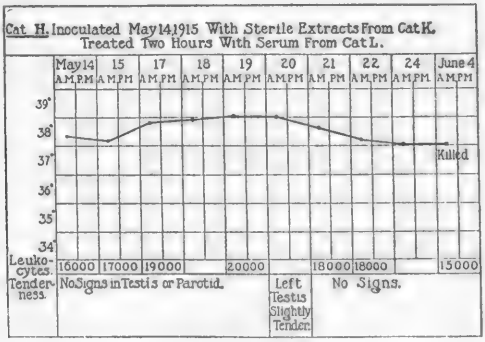
Cat H.—Inoculated May 14, 1915, with glycerinated material from Cat K, kept in contact with serum of Cat L for two hours at 37° C. (Protocol VIII, Text-fig. 7). Cat L was inoculated Feb. 6; well since Mar. 1; bled on May 12.

Left parotid inoculated with left parotid of Cat K.
" testis " " " testis " " "

PROTOCOL VIII.

Date.	Temperature.	Leukocytes.	Remarks.
1915	°C.		
May 14	38.3	16,000	Polynuclears 70; large mononuclears 12; small mononuclears 12; eosinophils 4.
" 15	38.2	17,000	No signs.
" 17	38.9	19,000	Polynuclears 74; large mononuclears 10; small mononuclears 13; eosinophils 3. No signs.
" 18	38.9	.....	
" 19	39.0	20,000	Polynuclears 69; large mononuclears 13; small mononuclears 14; eosinophils 4. No signs.
" 20	39.0	.....	Left testis slightly tender.
" 21	38.6	18,000	No signs.
" 22	38.2	18,000	Polynuclears 71; large mononuclears 14; small mononuclears 13; eosinophils 2. No signs.
" 24	38.0	.....	No signs.
June 4	38.0	15,000	" "

Chloroformed. Neither parotid nor testis showed any lesion on gross or microscopic examination.



TEXT-FIG. 7.

In comparing the case histories of the two cats, it will be noted that there are differences in the temperature and leukocyte counts, as well as in the reaction within the organs. Thus while the temperature of the control animal rose to 40° C., and remained above 39° for a week, the cat injected with the serum-treated material showed a rise to 39° C. over a period of only two days. Again, the

white cells of the control had increased to double their initial number on the eighth day after inoculation, and had not returned to normal ten days later; in the serum cat, on the contrary, they had risen only 4,000 in actual number by the fifth day, and the count had become practically normal within a week. The differential count remained quite unchanged in the second animal, while the control showed the early characteristic polynuclear cytosis and the later lymphocytosis.

The serum from a normal cat which had not been inoculated was employed in like manner to the immune serum, to serve as a serum control. The normal serum did not inhibit the action of the virus, as Protocol IX indicates.

Cat M.—Inoculated Dec. 15 with sterile extract of parotid and testis of an inoculated cat, which had been kept in contact with normal cat serum for 2 hours at 37°C. Right parotid and left testis injected.

PROTOCOL IX.

Date.	Temperature.	Leukocytes.	Remarks.
1915	°C.		
Dec. 15	38.9	21,000	Polynuclears 62; large mononuclears 19; small mononuclears 14; eosinophils 5.
" 16	38.9	23,000	No signs.
" 17	39.3	.....	
" 18	39.2	25,000	Polynuclears 65; large mononuclears 21; small mononuclears 12; eosinophils 1.
" 20	39.3	30,000	Left testis tender.
" 21	39.2	.....	" " more tender.
" 22	39.5	32,000	
" 23	39.7	.....	" " tender.
" 24	39.6	35,000	" " "
			Polynuclears 56; large mononuclears 18; small mononuclears 21; eosinophils 5.
			Chloroformed.

At autopsy the right parotid was distinctly swollen, and more congested than the left. The left testis was slightly larger than the right.

These experiments indicate that the serum of the cats employed has the power to reduce the development of the reaction produced by the injection of testicular and parotid materials derived from cats treated with filtrates of the saliva from cases of acute parotitis.

Blood Changes.

Daily blood counts were made on several normal cats, to ascertain the blood picture in healthy animals. It was found that the average number of leukocytes varied from 16,000 to 20,000, and that a difference of 5,000 from day to day was not unusual. It follows, therefore, that only a very decided increase in the number of white cells can have any significance in the cat.

The white cells began to increase within the first forty-eight hours after the inoculations, while on the seventh or eighth day a sudden additional rise, often doubling the initial count, may occur. The increase is maintained, with daily fluctuations, for a period of eight to ten days, the maximum being reached on about the fourteenth to the sixteenth day. The fall in number takes place gradually, the initial number being reached as a rule in about four weeks. During the first two weeks the leukocytosis is polymorphonuclear in character. At the end of the second or beginning of the third week, when the fever and glandular swellings are at the highest, a lymphocytosis is noted. The large mononuclear and eosinophil cells remained practically unchanged throughout.

The blood picture as described in the experiments is not very dissimilar from the one observed in cases of parotitis in man with testicular complications. Wile[9] has noted the change from a lymphocytosis to a polynuclear leukocytosis under these conditions. Apparently the change is inconstant, since Feiling[10] has not always found it. Recently Aubertin and Chabanier[11] have noted an initial polynuclear leukocytosis in all cases of mumps examined, the lymphocytosis following later, a fact which does not agree with Feiling's observations, which are to the effect that a relative and absolute lymphocytosis is present on the first day of epidemic parotitis. The increase in mononuclear cells found by Nicolle and Conseil in one of their monkeys, which had been inoculated into the parotid but not into the testicle, is of some interest in this connection, as is the polynuclear leukocytosis without the later appearance of a lymphocytosis

[9] Wile, I. S., *Arch. Pediat.*, 1906, xxii, 669.
[10] Feiling, A., *Quart. Jour. Med.*, 1915, viii, 255.
[11] Aubertin, Ch., and Chabanier, H., *Arch. d. mal. du coeur*, 1915, viii, 1.

noted in cats inoculated intratesticularly with material from normal cats.

That the polynuclear leukocytosis is not due to suppurative inflammation of the testes is indicated by the microscopic examinations, which showed also that at the time the testicles were removed no excess of polynuclear cells occurred in them.

DISCUSSION.

The object of the study described in the preceding pages was the reproduction of the chief organic lesions of parotitis in animals by means of filtered extracts of the saliva derived from persons suffering from acute parotitis. The experiments indicate that certain definite results have been obtained. It now appears that the injections into cats of bacteria-free filtrates of the saliva derived from cases of acute parotitis are capable of setting up a series of pathological changes in the parotids and testicles, expressed by fever, leukocytosis, tenderness, and swelling attended by definite histological alterations. Whether these changes accurately reproduce the condition arising in man in acute parotitis need not now be decided. The interest of the observations increases, however, in view of the fact that the effect of the injections is intensified by successive transfers of the inoculated organs from animal to animal through several passages.

Upon what principle present in the saliva the effects produced depend need not be discussed at the moment. It is obvious that ordinary bacteria can be excluded, since they were never found in microscopic preparations of the filtrate, or in aerobic or anaerobic (tissue) cultures, or in film preparations and sections made from the inoculated parotids and testes. On the other hand, it seems necessary to suppose that the active agent is of living nature; and thus the conclusion of a minute filterable virus is suggested.

The effects attributed to the saliva from cases of parotitis do not follow the injection of filtrates prepared with the saliva of normal persons. Moreover, and this point is a suggestive one, the active agent present in the saliva in cases of parotitis is neutralized or rendered ineffective by the serum of a cat which had been permitted to survive the injection of testicular and parotid emulsions, while the serum of a

normal cat had no such power. Assuming the activity of the salivary filtrate to be due to a living virus the power of prevention exercised by the serum may be assumed to be due to an immune body which was developed in the inoculated cat.

SUMMARY AND CONCLUSION.

Cats injected into the parotid gland and testicle with a bacterial sterile filtrate of the salivary secretion of children in the active stage of parotitis or mumps can be made to develop a pathological condition having several points of resemblance to the condition present in mumps in human beings.

After an incubation stage of from five to eight days definite changes have been noted in the temperature, blood leukocytes, and inoculated organs.

The temperature rise begins within twenty-four hours of the inoculations and reaches a maximum in from seven to fourteen days. The febrile rise fluctuates between 1° and 2.5° C.

The white blood cells begin to increase on the second day following the inoculation. The first change is a polymorphonuclear leukocytosis which precedes the glandular swellings. This initial rise is followed by a decline, after which the lymphocytes increase. The increase is confined to the small lymphocytes, which increase to from 7 to 10 per cent of their initial number.

The inoculated glands become swollen and tender. The swelling and tenderness become apparent from the fifth to the ninth days and persist for a variable period. The parotid changes are less constant or less obvious than are the testicular. The latter are constant and endure from eight to twelve days.

The rise of temperature and the leukocytosis precede the glandular swelling, but all the changes reach the maximum at about the same time, after which they decline gradually. What may be regarded as normal conditions are reestablished in four weeks or less.

The intraparotid and intratesticular injections of extracts of normal parotid gland and testicles may cause a mild rise of temperature and leukocytosis of brief duration, but swelling and tenderness are absent. The white cells increased are the polymorphonuclears and not the lymphocytes.

The intraparotid and intratesticular injections of filtrates of normal saliva may cause a mild rise of temperature of very brief duration, but leukocytosis, swelling, and tenderness do not appear.

The histological changes in the parotid gland when present consist chiefly of edema of the interlobular connective tissue with mononuclear interstitial infiltration about the ducts and elsewhere. In cases of long duration the ducts may be dilated. But in some instances the swollen gland while showing congestion and edema in gross showed inconspicuous changes under the microscope. The histological changes in the testicle are of two kinds: inconstant changes of cellular invasion between the tubules and swelling or even multiplication of the interstitial cells, constant ones consisting of degeneration of the epithelium and interference with spermatogenesis, a condition to which we have applied the term "spermatorrhexis."

The pathological conditions set up by the filtrate derived from the salivary secretion of cases of acute parotitis are intensified by successive transfers through a small series of cats of the extract and emulsion of the parotid gland and testicle previously inoculated.

The pathological changes are also prevented or reduced when the extract or emulsion is previously incubated with a quantity of blood serum obtained from a cat which has survived inoculation. Normal serum, on the other hand, has no such inhibiting effect.

The deduction from these experiments is to the effect that the salivary secretion in parotitis or mumps contains a filterable substance capable of setting up a series of definite pathological conditions when inoculated into the testicle and parotid glands of cats. Whether this active material is a microorganism and if so whether it is the specific microbic cause of parotitis or mumps remains to be ascertained.

EXPLANATION OF PLATES.

PLATE 53.

FIG. 1. Parotid gland, showing cellular infiltration around the secretory ducts. Cat killed after 18 days.

FIG. 2. Parotid gland, showing cellular infiltration and dilatation of the secretory ducts. Cat killed after 17 days.

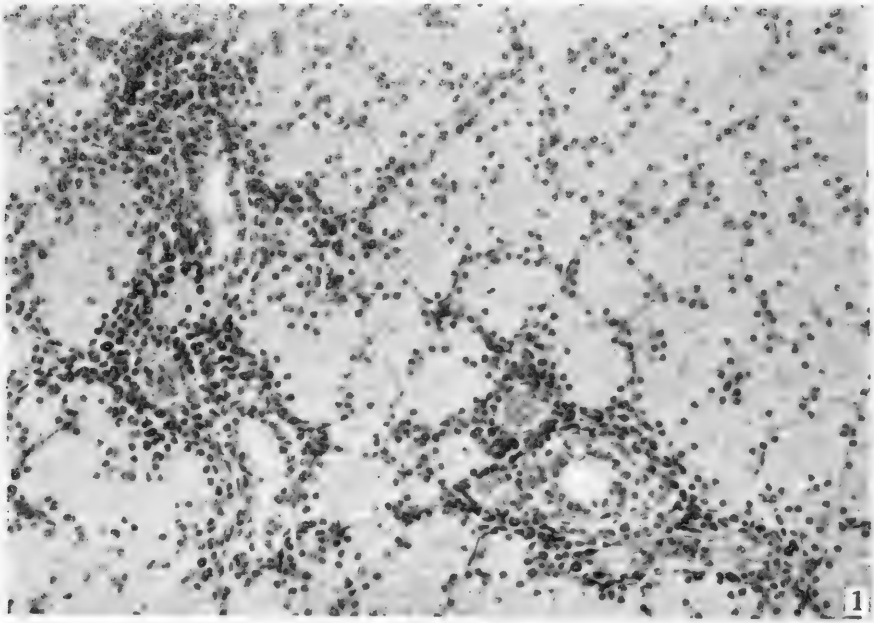
PLATE 54.

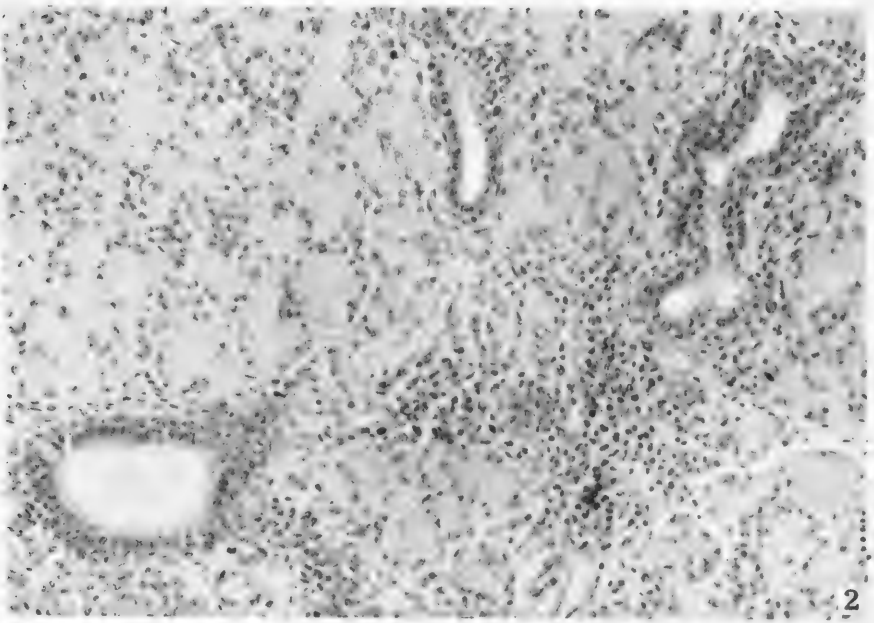
FIG. 3. Testicle, showing cellular infiltration around the tubules, and spermatorrhexis. Cat killed after 12 days.

FIG. 4. Testicle, showing increase in the size and number of the interstitial cells, and spermatorrhexis. Cat killed after 5 weeks.

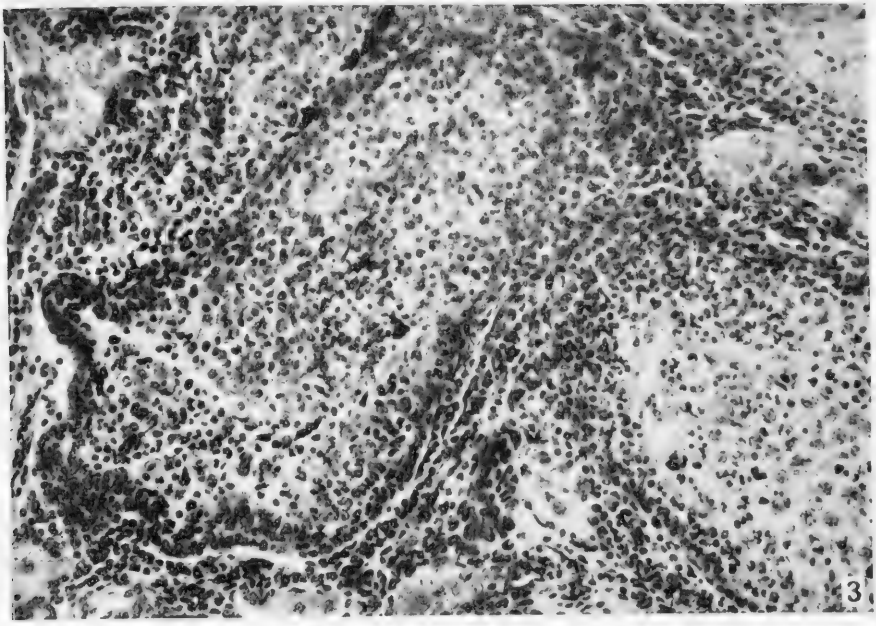
PLATE 55.

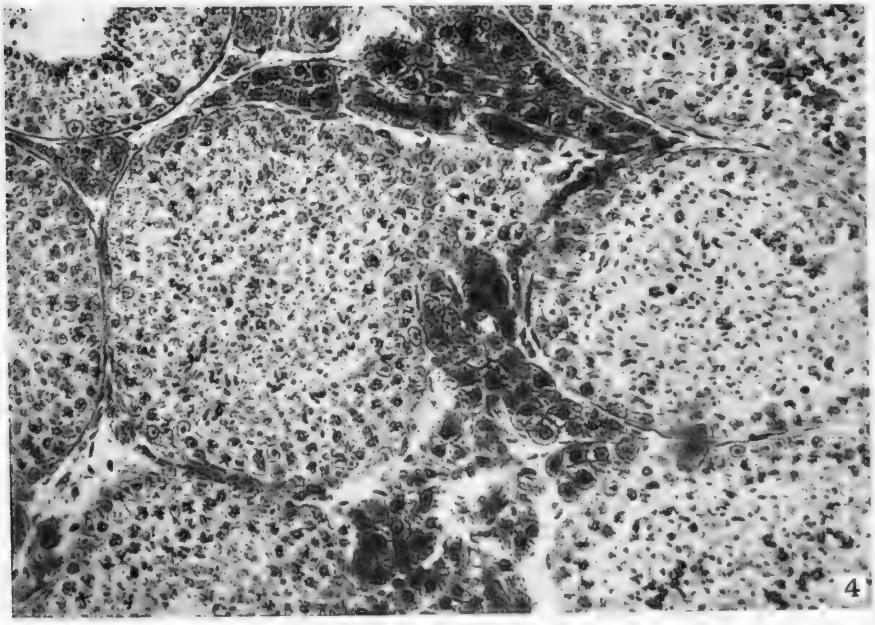
FIG. 5. Parotid glands, showing swelling of the inoculated (left) side at autopsy. Cat killed after 13 days.



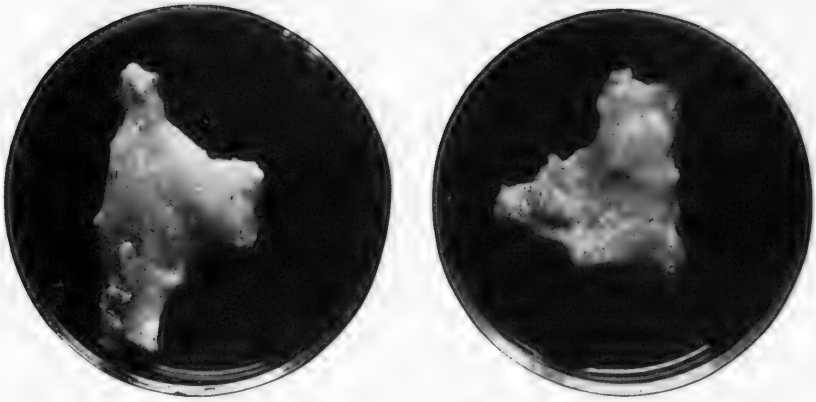


(Wollstein: Experimental Study of Parotitis (Mumps).)





(Wollstein: Experimental Study of Parotitis (Mumps).)



Left parotid, inoculated. Right parotid, not inoculated.

FIG. 5.

(Wollstein: Experimental Study of Parotitis (Mumps).)

THE ETIOLOGY, MODE OF INFECTION, AND SPECIFIC THERAPY OF WEIL'S DISEASE (SPIROCHÆTOSIS ICTEROHÆMORRHAGICA).

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Plates 56 to 62.

(Received for publication, December 1, 1915.)

INTRODUCTION.

In this communication we have summarized and in some details extended the publications on this subject which have appeared in the Japanese literature.[1] In the course of our investigations of that endemic disease of portions of Japan, which agrees clinically with Weil's disease, so called, we discovered a spirochætal microorganism which is now believed to be the cause of the disease. In the experiments recorded in this paper, Drs. Inada, Ido, Hoki, and Ito chiefly conducted the experiments on animals, and Dr. Kaneko the pathological and anatomical observations.

In the western part of Japan there prevails an epidemic and endemic disease characterized by conjunctival congestion, muscular pain, fever, jaundice, hemorrhagic diathesis, and albuminuria, which is known as Weil's disease or febrile jaundice. A similar disease is also present in Shikoku and is called *Odan-eki;* that is, icteric pestilence. At the end of last year (1914) the same disease was observed in Chiba, in the eastern part of Japan, near Tokyo, where the patients numbered 178. Thus this disease appears to be prevalent in various parts of Japan, although to a small extent.

We have been interested in Weil's disease in Kyushu for many years, and at the end of November, 1914, we detected a spirochæta in the liver of a guinea pig injected with the blood of a patient suffering from Weil's disease. We came to the conclusion, in January, 1915, that this spirochæta is the pathogenic cause of Weil's disease, and we named it *Spirochæta icterohæmorrhagiæ*. At Chiba, a distance of more than 700 miles from Kyushu, we detected in the blood of five out of

[1] A full bibliography is appended.

six patients who suffered from this disease in 1914 a substance which has a spirochæticidal power over this spirochæta. Nishida, a physician in Kusunoki Hospital in Kochi, Shikoku, a former pupil of Inada, also detected by our method, a spirochæta in a patient suffering from *Odan-eki*, and his spirochæta was morphologically identical with ours.

From these facts we were able to identify the pathogenic cause of Weil's disease prevailing in three different parts of Japan, that is, in Kyushu and Chiba, and *Odan-eki* in Kochi, Shikoku, as *Spirochæta icterohæmorrhagiæ*. We can now say that it is an independent disease, caused by a specific microorganism, and we therefore propose to substitute the name of spirochætosis icterohæmorrhagica for the various names already in use. This name, although somewhat inconvenient, expresses both the symptoms and cause of the disease. Thus, some patients suffering from Weil's disease exhibit rarely only slight jaundice or do not show hemorrhage, and for these cases the name may seem inappropriate; but the occurrence of such light and atypical cases is a common experience among infectious diseases. We might shorten the name to spirochætosis icterica, as the jaundice is the most striking and constant symptom, but there is danger of confusing it with recurrent fever accompanied by jaundice; while, moreover, the name spirochætosis japonica does not differentiate it from other diseases caused by various spirochætæ.

Opinions differ as to whether Weil's disease in Europe and Japan are identical. The symptoms are similar, but there are some variations. The mortality of Weil's disease is 32 per cent in our clinic; splenic enlargement is rare (10 per cent in our clinic). Spirochætosis icterohæmorrhagica is present endemically in various localities, but rarely causes a sudden epidemic, as is the case with the European Weil's disease. On the other hand, the European Weil's disease appears either as a transient epidemic or endemically, and in the latter case the state of its outbreak and symptoms resembles the Japanese disease closely. Thus the above question can only be solved by the detection of the presence or absence of the spirochæta in the European Weil's disease.

Bilious typhoid fever resembles the Japanese Weil's disease. According to Griesinger and others, this disease is a form of recurrent fever accompanied by jaundice; but Kartulis, Diamantopulos, Goldhorn, and Rabinowitsch believe that it has no connection with recurrent fever. Goldhorn states that Weil's disease is a light form of bilious typhoid, and Weil himself called attention to the similarity of the symptoms in the two conditions. Fiedler reports that pathological anatomical changes in Weil's disease are the same as those in bilious

typhoid. The mortality of bilious typhoid, according to Griesinger, is 60 to 70 per cent; compared with that of our cases of so called Weil's disease this is high. A few years ago Nishi identified Weil's disease with yellow fever, but he was opposed by Ohno. We have also emphasized the differences from the epidemiological point of view between yellow fever and Weil's disease, although the symptoms are very much alike. The poison in Weil's disease is sometimes capable of filtration through a Berkefeld filter, as is that of yellow fever, and the microbic causes of both diseases are present in the blood only in the early stage of the illness. Both diseases are prevalent in the lowlands. Yellow fever, however, is communicated to man by the *Stegomyia* mosquito, while here is at present no proof that Weil's disease is communicated by the bite of an insect.

Typical Japanese Weil's disease can be easily differentiated from catarrhal jaundice. On the other hand, very light cases or cases in which the conjunctival congestion has already passed or which have passed beyond the initial stages and show no hemorrhages may be difficult to diagnose. Clinically one never sees in catarrhal jaundice such marked congestion of the conjunctiva as is present in the Japanese Weil's disease, and the general condition of the patients shows great differences in the two diseases. In Weil's disease the patient is weak and cannot stand up even on the 1st or 2nd day of the illness, while in catarrhal jaundice one does not meet with such symptoms. The state of the circulatory system and other symptoms make further differentiation possible. However, the two diseases may not be differentiated through the presence or absence of bactericidal substances in the blood of the patients.

Spirochæta icterohæmorrhagiæ.[2]

In February, 1915, we announced the discovery of the spirochæta of Weil's disease. It may be interesting, therefore, to review briefly the reasons for the failure to detect the spirochæta at an earlier period: (1) All investigators searched for bacteria. (2) The spirochæta resides in the blood only during the early stages of the disease and is present there in small numbers. (3) At the period at which fatal cases come to autopsy the liver is either devoid of spirochætæ or they are so few or modified in form as to be difficult of discovery or recognition.

In our early studies we searched for bacteria in the blood, urine, and feces. Having failed in this quest, we inoculated the monkey, rabbit, rat, and guinea pig with the blood of patients, and in July, 1913, we observed that the guinea pig developed albuminuria, conjunctival congestion, jaundice, and hemorrhages when the blood was

[2] This part of the investigation was conducted by Drs. Inada and Ido.

injected during the first 7 days of the illness. We ascertained later that the number of spirochætæ in the blood is so small as to make their detection under the microscope almost impossible. In the guinea pig they multiply freely and are found in large numbers in the liver. We first detected the spirochætæ in this manner in November, 1914. Inasmuch as the injection of the blood of healthy persons or even of patients suffering from other diseases including catarrhal jaundice does not cause pathologic changes in the guinea pig or lead to the occurrence of spirochætæ in the liver, we concluded that the spirochætæ were derived from the blood of the cases of Weil's disease. Our deductions were confirmed when we discovered the same spirochætæ microscopically in six specimens of patients' blood, in the intestinal wall of one case, and in the adrenal glands of another among eleven autopsied cases. The autopsies were performed on cases which had succumbed on the 8th to the 14th day of illness, the most fatal period of the disease. At this period the spirochætæ have already diminished greatly in numbers. At this time we also detected a bactericidal substance for the spirochætæ in the blood of patients, and we assumed that the disappearance of the organisms from the body was connected with the formation of this substance. Probably in this way is explained either the entire absence or the occurrence merely of atypical forms in small numbers in the organs. On the other hand, we detected later in the liver of two patients who succumbed on the 6th day of illness spirochætæ as numerous as in the liver of the guinea pig. These facts led us to assert that the spirochæta is the pathogenic cause of Weil's disease.

EXPERIMENTAL.[3]

Hecker and Otto injected defibrinated blood of soldiers suffering from Weil's disease in Europe into the skin, peritoneum, and veins of rats, guinea pigs, rabbits, and monkeys, with negative effects. Fiedler and Schittenhelm report similar results. We have injected blood of seventeen cases of spirochætosis icterohæmorrhagica into the peritoneum of the guinea pig. Thirteen positive results with typical symptoms,—conjunctival congestion, jaundice, hemorrhage, and albu-

[3] These experiments were carried out by Drs. Inada, Ido, and Hoki.

minuria,—were obtained. The blood of all the patients in the 4th to the 5th day of the illness gave positive results, while that taken on the 7th day in one instance gave positive and in another negative results. The blood in one case, on the 9th day of illness, also gave a positive result; but in no instance from the 12th day on was a result obtained, although blood from three such cases was tested. Thus, in order to obtain positive results one must inject the blood within the first 7 days of the illness. The symptoms following the injection are similar to those after inoculation from one guinea pig to another, except that the incubation period is longer. The incubation period may be reduced by making the inoculation from a patient suffering from severe illness in its early stage.

The time required after the inoculation for the appearance of jaundice is usually 7 to 8 days. The shortest time was 6 days, and the longest 13 days. The infection can be transferred from an infected guinea pig to others through many generations, and the oldest of our six strains of spirochæta has now reached the fifty-first generation (July 15, 1915). The inoculation succeeds by intraperitoneal, subcutaneous, or oral injection of 2 cc. of the heart's blood or of an emulsion of the liver. The former is the most certain. The method of inoculation also affects the incubation period. The animals succumb in from 5 to 8 days after intraperitoneal, and in from 9 to 10 days after inoculation through the uninjured skin or the alimentary canal; after inoculation into the injured skin death follows in 7 to 8 days. The spirochæta occurred in the animals experimented upon practically constantly. Of the large number of experiments made, in only two were they discovered in the liver, although jaundice and hemorrhage were present.

The symptoms produced in the guinea pig by inoculation are similar to those occurring in man. The animals show loss of appetite, conjunctival congestion, anemia, jaundice, hemorrhagic diathesis, and albuminuria. The fever comes on suddenly, reaching nearly 40°C. on the 4th or 5th day after intraperitoneal inoculation, and on the 5th or 6th day after the inoculation through the injured skin. The spirochæta appears in the blood about this time and the animals generally succumb within 24 hours after the appearance of the jaundice. Just before death the temperature falls rapidly, as in collapse.

Rabbits fail to become infected even when large quantities of the blood capable of producing the disease in guinea pigs are injected. In our experiments,

death never followed the intraperitoneal or intravenous inoculation of 20 to 40 cc. of blood or liver emulsion of the guinea pig containing the spirochæta, the fatal dose for the guinea pig being 4 to 5 cc. However, eleven of the thirteen rabbits showed a rise of temperature on the 4th or 5th day. Conjunctival congestion was present in six, and a slight jaundice in four animals. All the animals became excited. One which showed marked jaundice showed no hemorrhages or spirochætæ in the liver.

Fourteen mice were inoculated with the liver emulsion or blood of the infected guinea pig, of which four showed jaundice and slight hemorrhage and succumbed. One was examined, and no spirochætæ were found in the liver.

Two white rats were similarly inoculated. One developed jaundice and slight hemorrhage and died on the 8th day; the other remained healthy.

From the above experiments the rabbit appears to be the most insusceptible, the mouse and white rat coming next. The guinea pig is the most susceptible; it appears to be even more susceptible than man.

Pathological Changes in the Guinea Pig.[4]

The typical pathological changes consist of marked general jaundice, hemorrhages into the various parts of the body, and parenchymatous changes of the organs. The usual sites of hemorrhage are the lungs, intestinal walls, retroperitoneal tissues, and the fatty tissue of the inguinal region. When the changes are atypical there are either very slight or no hemorrhages, although the jaundice is marked, or there is no jaundice although hemorrhage is present. The latter condition is rare and generally when met with the spirochætæ are few in number or absent. After the injection of salvarsan this condition is found.

The liver shows cloudy swelling of the parenchyma, while the color varies according to the degree of the jaundice and the quantity of blood present. Microscopically the precipitation of the bile is not marked in spite of the presence of jaundice, and there is no congestion of the bile in the biliary duct.

The kidney shows an acute parenchymatous nephritis. The spleen is more or less enlarged, congested, and hemorrhagic. There is always some precipitation of the iron-containing pigment and the phagocytic cells contain red blood corpuscles. The lymphatic glands do not undergo enlargement, except those near the site of inoculation.

[4] This study was made by Drs. Inada and Kaneko.

The lungs present small and large hemorrhagic spots, like the wing of a mottled butterfly. This change is one of the most important in the diagnosis of the disease.

The intestines show hemorrhages in the walls. The muscles show a certain amount of hemorrhage and parenchymatous changes of the muscular fibers.

Distribution of the Spirochætæ.

The spirochæta lives in the blood outside the cellular elements and in various organs and tissues. When present in the cells it is within phagocytic cells. It is often seen in the epithelial cells. The liver contains the largest number of spirochætæ. They can be demonstrated most clearly by the older Levaditi method of silver impregnation. They occur usually in the spaces between the cells, and when present in large numbers are arranged about the individual cells like a garland. Next to the liver the adrenal glands and the kidneys contain the largest number of spirochætæ. In the kidneys the spirochæta occurs inside the interstitial tissues and also in the walls and lumen of the uriniferous tubules.

There are very few spirochætæ in the spleen, bone marrow, and lymph glands, although they are rich in blood; and the spirochætæ are extremely rare in the splenic follicles, as well as in the lymph glands and other lymphoid tissues. Diffuse hemorrhages in the lung and intestinal walls may contain only a small number.

With a given strain of spirochæta, the animals that show the most marked pathological changes have the most spirochætæ. Among the six strains of spirochætæ, two, which were obtained from severe cases, produced severe pathological changes associated with more numerous spirochætæ than followed the inoculation of the other strains. The pathological changes produced by the above two strains have, however, become gradually reduced as successive transfers have been made.

Comparison of the pathological changes in the guinea pig with those of man brings out certain differences: (1) The hemorrhage is severer and more wide-spread in the lungs of the guinea pig than in man. (2) Spirochætæ can always be found in the guinea pig when the inoculation gives a typical result, which is not the case in man. This

is the most important difference between the human disease and the infection in guinea pigs. (3) The spirochætæ are present within the liver cells in man, and lie between the liver cells in the guinea pig. This difference may be accounted for by the fact that no immunizing substance is formed in the guinea pig. In man a bactericidal substance injurious to the spirochæta appears in the blood on the 14th or 15th day of illness. Moreover, man is more subject to intercurrent mixed infections than the guinea pig.

Characteristics of the Spirochætæ.[5]

Spirochæta icterohæmorrhagiæ remains always outside the blood cells. It resides in the interstitial tissues of the organs and rarely in epithelial or phagocytic cells. Like that of recurrent fever it probably belongs to the blood spirochætæ. This view is supported by the acute nature of the disease and by the fact that the spirochætæ of chronic diseases like syphilis and frambesia belong to the group of tissue parasites. Although it is impossible to measure the length of the spirochæta exactly, because of its irregular wavy figure, the specimens seen in blood taken from a case of Weil's disease varied from half the diameter to the full diameter of the red corpuscles. The measurements are the same in the blood of the guinea pig. The commonest length is between 6 to 9 μ, the greatest reaching 12 to 13 μ. In the liver the short forms measure from 4 to 5 μ; but the common forms are longer and measure 8 to 9 μ. The longest individuals average 20 μ, although we have seen one measuring 25 μ.

The thickness varies according to the staining and fixing methods and the strength of the stain. The thickness is probably 0.25 μ. The ends are sharp and in most cases hooked. In some specimens one end only is hooked, and in others both ends are bent toward the same side, resembling the letter C; or the ends are turned opposite to each other forming the letter S. The undulations are not so regular as in *Treponema pallidum* and are usually composed of two or three large irregular, or four or five smaller waves.

Flagella cannot be demonstrated by Loeffler's method, and it is probable, therefore, that a membrane is absent. This point is not established.

[5] This study was made by Drs. Inada and Ido.

The methods employed in staining are as follows: Liver and blood specimens are fixed with absolute alcohol, methyl alcohol, or osmic acid (Weidenreich's method), and the staining is done with Giemsa's solution. The specimen fixed with methyl alcohol is stained for 2 hours or over with a mixture of about 2 cc. of water and three drops of Giemsa's solution. The spirochæta is usually well stained when the granules of the white blood corpuscles are deeply stained. The color of the spirochæta varies according to the degree of the staining, and is either red or red with a purplish tinge. For vital staining 50 per cent borax methylene blue is employed.

The spirochætæ stained in this manner look uniform, but under high magnification some individuals show folds and present the granular appearance seen under dark-field illumination. Moreover, some of the spirochætæ show the granular appearance distinctly when stained by Loeffler's method.

The spirochætæ in the blood usually show the typical and rarely the atypical form. But when they are very numerous, the atypical forms—ring form or two twined about one another—are more frequent.

The forms present in the liver are as variable as are the differences in length. One sees round or oblong granules, sometimes three or four in number, stained deep purple with Giemsa's solution in the body of the spirochæta. These granules appear to be collections of chromatin. In addition still larger granules sometimes project from the body of the organism forming the so called bud of the spirochæta. As in the case of the other spirochætæ, the significance of this bud is not apparent. Some of the spirochætæ resemble a platinum loop at one end or appear as rings, in which case the ends are not visible. The degenerative form is thick and straight, devoid of waves, and blunt at the ends.

The spirochætæ are not visible in the unstained condition under the microscope, even when very numerous. Under dark-field illumination they are readily found and present a characteristic appearance. The light is refracted unevenly, and portions showing strong light alternate with portions showing no light. The latter are narrower than the former, giving to the organism the appearance of a rosary. According to the length of the spirochæta the refractive

granules number 25 to 30 to 40. There are fore and aft movements besides movements of the ends to right and left. The anterior third of the spirochæta makes brisk movements to the right and left, while the posterior part makes only slow movements with the end turned either to the right or left. There is also a twisting motion about the long axis, which makes the spirochæta look like a figure 8, and there are spiral or snake-like movements, besides the formation of waves along the long axis, as if the waves of muscular contraction were transmitted to the spirochæta.

When a liver emulsion is left at room temperature, the spirochæta keeps its movements for 2 days, although they are not brisk, at the end of which period the majority are precipitated to the bottom of the culture tube.

The spirochæta immediately ceases to move when put in 50 per cent glycerine, 5 per cent sodium chloride, or 0.5 per cent acetic acid solution; it looks as if it were hardened and shows various degenerative changes, such as a bending small ring formation at one or both ends. 0.5 per cent sodium hydrate solution immediately dissolves the spirochætæ, but they continue to move for a comparatively long time in distilled water.

Filterability of the Spirochætæ.

The results of experiments conducted with five Berkefeld V, one N, and three W candles were variable. The filters had previously been tested with *Bacterium coli commune*, not with *Bacillus prodigiosus*, and were found to be impervious to the former. Some of the filtrates when injected produced infection in a guinea pig, but spirochætæ were not detected under dark-field illumination.

Of twenty-eight experiments made with liver emulsion of a particular strain, fifteen positive results were obtained. As a rule, the spirochæta appears to be more easily filterable when present in large numbers.

It is difficult to determine the mode of multiplication of the spirochæta, and various opinions might be expressed, as with the mode of the multiplication of other spirochætæ. We examined many fresh specimens under dark-field illumination daily for over 6 months. On one occasion Hoki saw a spirochæta suggesting transverse seg-

mentation, and Ito and Inada saw one suggesting longitudinal segmentation. We think, however, that multiplication probably occurs by transverse segmentation, since one sees numerous short forms at times when the multiplication of the spirochæta is going on rapidly, as is described below.

Cultivation of the Spirochætæ.[6]

It was not until April, 1915, that we succeeded in keeping the spirochæta alive from 13 to 17 days. In May we observed its multiplication and were able to transmit it from generation to generation. At that time we cultivated it through five generations, and the spirochæta lived in the culture medium for 2 months after it left the body of the animal. The method employed was that of Noguchi, by means of which various spirochætæ of recurrent fever have been cultivated. We employed guinea pig instead of rabbit kidney, and always used liquid paraffin. The most important fact, however, is that the temperature of 37°C. is not suitable for its development. When a well grown culture is kept at 37°C. the movements of the spirochæta become sluggish within 2 to 3 days, and almost all the spirochætæ undergo degeneration and finally disappear from the culture medium. Temperatures below 15°C. are also unsuitable, the best results being secured at temperatures of 22 to 25°C.

The culture does not yield any odor, and the ascitic fluid remains uncoagulated. Moreover, the fluid remains clear; and even slight cloudiness indicates contamination with some coccus or bacillus. The spirochætæ are distributed almost uniformly throughout the culture medium. The life of the culture is variable at 22–25°C. The first generation lives mostly from 3 to 6 weeks, the longest period observed being 55 days and the shortest 17 days. The life of the second and third generations is somewhat shorter than that of the first generation. The conditions which conduce to long life are at present not established. When extraneous bacteria are present in considerable numbers in the culture the spirochætæ die, but they can survive if the number of foreign bacteria is small, although they will not grow when transplanted. Thus protection from contamina-

[6] This work was done by Drs. Inada and Ido.

tion is an important factor in the cultivation. The multiplication of the spirochætæ in the culture starts at different periods, sometimes after 2 to 3 days and at other times only after 1 week or, rarely, after 2 weeks. In the transfer of the culture from one tube to another the addition of a small quantity of blood keeps up the development, while the best time for the transfer is when multiplication is going on rapidly, as is indicated by an examination of the culture every 2 or 3 days.

In character the cultivated spirochæta does not differ from that obtained directly from the animal body. When multiplication is at its height the spirochæta is very short and has brisk movements. In the young culture over half the spirochætæ may be composed of the short forms which, however, gradually become longer. Sometimes two spirochætæ are connected, and again one sees 8 to 9 to 15 spirochætæ collected about some granular substance in a manner resembling a rosette, with very brisk movements. Rarely very long spirochætæ (two or three times the normal length) composed of a single organism may occur. After the height of development the movements gradually become sluggish, the spirochæta takes on a hardened and bent appearance, assumes various degenerative forms, and finally dies. The second and third generations of the pure culture are capable of producing on inoculation infection in the guinea pig; whether later generations do so has yet to be determined. In the absence of a simpler method, we have successfully employed Noguchi's method of cultivation.

Mode of Infection.[7]

Weil believed that in the European Weil's disease infection occurred through the alimentary canal. Fiedler held the same view, but Hecker and Otto believed that infection might be communicated by the bite of a mosquito. As regards Weil's disease in Japan, Inada thinks that the infection occurs probably through the alimentary canal. It may, however, sometimes enter through the skin, as at times the disease begins with local swelling of the lymph glands. The throat is probably not the place of invasion, although it may show congestion. Oguro in Saga, on the other hand, has never observed

[7] This study was made by Drs. Ido and Hoki.

any circumstance which suggests the cutaneous mode of infection, but regards the alimentary tract as the portal of infection. Thus the mode of infection cannot be considered as established from clinical observation. On the other hand, since the discovery of the pathogenic cause of the disease, we have been able to determine it by experiments on animals.

The abdominal wall of the guinea pig is shaved without injuring the skin, washed with soap and then with alcohol, and dried. 1 cc. of the liver emulsion containing the spirochæta is dropped on its surface with or without abrading it. Thirty guinea pigs were so treated after incision of the abdominal skin to the extent of causing slight bleeding, and thirteen animals without injuring the skin. 10 of the latter 13 animals (77 per cent) and 26 of the first 30 (86 per cent) contracted the disease. Thus one can see that the spirochæta is able to penetrate through a macroscopically healthy skin and cause the disease in the guinea pig. The invasion occurs more easily and certainly where an obvious lesion exists. The incubation period, moreover, is 9 to 10 days in the former, and 7 to 8 days in the latter instances. Crushed liver acts as well as the emulsion for the purpose. The time required by the spirochæta to penetrate through the skin is short, and even after the skin is washed with alcohol or sublimate solution, the animals acquire the disease 5 minutes after the application of the emulsion to the skin. Whether penetration takes place in less than 5 minutes has not been determined. The spirochæta can also be made to invade the body through the alimentary canal. Feeding 2 gm. of liver emulsion, or giving an enema of 2 gm. of liver emulsion containing the spirochæta, produces the disease in the guinea pig. Thus, the spirochæta is able to invade animals through the mucous membrane of the alimentary canal.

Among fifty-five cases which were admitted to our clinic, only a few indicated cutaneous origin. The following facts, however, suggest this mode of infection: (1) When the disease occurs in coal mines the patients are numerous among the miners who work at a certain part of the mines. Numerous cases do not arise in the same barracks, even when the disease originates there. (2) The clerks working outside the mines do not contract the disease. (3) There are many cases in wet mines and few in dry mines. (4) It was noted that many cases occurred among the miners who worked in a particular part of the mine, but no case occurred when the accumulated water, as suggested by Inada, was pumped out. As mentioned above, the spirochæta is able to penetrate a healthy skin. Thus anyone who is working in an infected locality can easily contract the dis-

ease, even when the skin is uninjured, and the infection will take place still more easily if the skin is injured. Coal miners, who are liable to abrasion of the skin, and also to a skin lesion caused by working with the feet in water, can easily contract the disease.

We thought that infection through the skin was probably on account of the presence of enlarged lymph glands, but, later, after observing numerous cases, we considered the enlargement of the glands as a result of the general infection and not the result of the direct invasion of the spirochæta into the glands. However, from the animal experiments described above, we believe that the enlargement is partly due to the result of local infection of the glands in the affected part and partly to the general infection. The enlargement of the glands is not a symptom which is present in every case; it was present in about 60 per cent of fifty-five cases admitted to our clinic. The enlarged glands usually reach the size of a horse-bean or less. We have only once seen the gland as large as the end of the thumb. Besides skin infection, infection through the alimentary canal possibly occurs also. Cases arise in which two or three persons in a family are affected at the same time or at an interval of 1 or 2 days. However, we do not yet know how the infection in these instances occurs.

Since the origin of the infection through the skin is established, it is necessary to consider the question of whether the mosquito or flea may play a part in causing it. Among our clinical cases indication of direct infection from man to man is rare; while the relation of the seasons and the fact that the disease is common in Hakata and Chiyomachi and rare in Fukuoka on the other side of the river, make us think that the mosquito and flea do not play a part in the propagation of the disease.

The spirochæta of the Japanese Weil's disease is not present in such large numbers in the blood as is the spirochæta of recurrent fever; the number diminishes rapidly and they disappear comparatively soon from the blood. Moreover, the virulence in man is less than in the guinea pig, and wide-spread epidemics, as in recurrent fever, do not occur.

Mode of Excretion of the Spirochæta.[8]

In order to detect the mode of excretion of the spirochæta, it is necessary first to examine the excretions with dark-field illumination and to inoculate guinea pigs with them. This double procedure is important, for even when the spirochæta cannot be recognized with the ultramicroscope, the animal experiments may prove positive. Ida, of the Second Medical Clinic of Kyushu University, inoculated urine from a case of Weil's disease into a guinea pig and applied some of the urine to the skin of the animal, after injuring the surface. He obtained a positive result. Later we visited several coal mines and examined the excretions under the dark-field microscope. We conducted experiments with animals at various stages of the disease with twenty-four cases.

In twelve cases we examined the urine with the dark-field microscope within the first 10 days of illness, and recognized a small number of spirochætæ, in one case on the 6th and in another on the 10th day. The others gave negative results.

It is noteworthy that numerous spirochætæ are found in the urine at a time when the immune body appears in the blood; that is, about the 13th to the 15th day of illness. In seven cases from the 10th to the 30th day of illness, we found numerous spirochætæ in the urinary sediments of the five patients in which a negative result was obtained during the first 10 days. In four of these five patients the microscopic field contained countless spirochætæ. The spirochætæ were chiefly present in the cylindroids and nubeculæ, and in small numbers in the cylinders. We studied the spirochætæ in two of these five cases and found that they begin to degenerate and finally disappear from the urine altogether before the 40th day of illness. Later we studied the urine from ten cases after the 30th day of illness and detected a small number of degenerative spirochætæ in two on the 32nd and 38th days, respectively. The remaining eight cases gave negative results.

Hence the urine contains the spirochæta, beginning at an early stage of the disease, but in such small numbers as to make detection by the dark-field microscope difficult. However, from the 13th to

[8] This work was done by Drs. Ido, Hoki, and Ito.

the 15th day of illness, at the time that the immune substance appears in the blood, they become numerous and are easily discovered. After the 24th to the 25th day the spirochætæ begin to diminish in number and to assume a degenerative form, while, in our experience, they disappear completely from the urine before the 40th day of illness. However, these points require further investigation before a definite conclusion can be reached.

The results of the inoculation of urine into guinea pigs are given in Table I.

TABLE I.

Material used for inoculation.	No. of inoculations.	Positive result.
Urine from 5 patients before the 10th day of illness.....	9	3
Urine from 4 patients between the 11th and 20th days of illness.....	15	5
Urine from 9 patients after the 31st day of illness.....	12	0

The inoculation of the urine taken during the first 10 days of illness gave results in one-third of the cases, even though it was difficult to detect the spirochæta in the urine; and, conversely, although the urine contained many spirochætæ after the 13th to the 15th day of illness, the inoculations gave only the same percentage of positive results. Possibly the immune substance, which begins to appear in the urine about the 24th or 25th day, may bear upon that phenomenon.

Feces from eight patients were inoculated into guinea pigs on ten different occasions with one positive result, from a patient on the 7th day of illness.

Sputum from two patients was injected into guinea pigs on three occasions. A positive result was obtained once in a case in which the sputum was bloody. Two inoculations with vomitus gave no result.

Manner of Excretion in the Experimental Disease.

On examination of the urine, feces, and the contents of the gall-bladder in twenty-five infected guinea pigs, a small number of spirochætæ was detected in the urine by the dark-field apparatus in eight

of the thirty-two examinations. The examinations of the contents of the gall-bladder were all negative; while those of the feces and intestinal contents gave microscopically a positive result in one of twenty examinations.

The inoculation test with the materials mentioned gave a wholly different result: the urine produced infection in 7 of 9 tests, the feces and the intestinal contents in 7 of 11 tests, and the contents of the gall-bladder in 2 of 3 tests. Thus, the spirochæta is excreted in the urine, feces, and bile in the experimental disease of the guinea pig up to the time of death, although the number excreted in the urine is small.

The following experiments were made in order to determine whether the excretion of numerous spirochætæ in the patient's urine in the convalescent stage of the disease is related to the appearance of the immune substance in the blood.

Experiment 1.—The distribution of the spirochætæ in the various organs was studied after immune goat serum was injected into the guinea pig at the time of the appearance of jaundice.

Experiment 2.—The urine from the guinea pigs which contracted the disease from cutaneous inoculation and which had recovered without developing jaundice under treatment with immune goat serum, was examined daily at the time when the spirochætæ were present in the blood. The animals were killed about 20 days after the injection of the immune serum, and the distribution of the spirochætæ in the body was studied.

The first experiment was limited to two animals, and no spirochætæ could be discovered in the liver under the dark-field microscope, although the kidney showed a small number.

The second experiment was performed with four animals. In two that passed the 10th and 13th days respectively after the injection of the immune serum, the spirochæta was recognized in the urine. All four animals were killed on the 20th day after the serum treatment, and the blood, liver, kidneys, and urine were examined under dark-field illumination. The blood and liver showed no spirochætæ, while the kidneys and urine in the bladder showed typical spirochætæ with active movements.

In the experimentally infected guinea pigs the number of the spirochætæ excreted in the urine is usually small, and this is probably due to the circumstance that the animals die before the immune substance is formed in the body. This is indicated by the fact that the spirochætæ appear in the urine in the animals which are treated with the immune serum. The serum treatment probably confers an immunity upon the animals.

Whether the formation of the immune substances which appear to limit the development of the spirochætæ in the body and cause their collection in the kidneys and hence excretion into the urine is responsible for the appearance of the large numbers in the urine in human cases at the time of convalescence, is not clear. Perhaps the spirochætæ are merely able to multiply in the kidney for a time, after which they are excreted. The autopsies indicate that these phenomena occur. The immune substances, moreover, are present in the urine as well as in the blood for a certain period after the 24th or 25th day of illness. When the immune bodies begin to appear in the urine the spirochætæ gradually degenerate and disappear.

Immunity Phenomena.[9]

In the spirochætal disease under consideration a substance which has bactericidal and bacteriolytic action over the spirochætæ appears in the blood. The discovery of this substance was of great importance in the detection of the spirochætæ. The existence of the immune body can be demonstrated by Pfeiffer's method, as follows:

1 to 2 cc. of liver emulsion, containing spirochætæ, are injected into the peritoneal cavity of guinea pigs. In one series of animals the patient's serum is also injected, while in the control animal salt solution or the serum of a healthy person is employed for the injection. After ½ to 2 hours peritoneal fluid is withdrawn and examined microscopically. The fluid from the control animals shows numerous spirochætæ with active movements, although in somewhat diminished numbers after 2 hours, while after half an hour the fluid from the animal treated with the patient's serum will be free of spirochætæ.

Moreover, the subsequent condition of the animals confirms the presence of the immune body. Those in which the spirochætæ disappear from the peritoneal fluid after half an hour usually do not develop the infection, while those which still show after 2 hours a small number of spirochætæ in the peritoneal fluid develop jaundice and hemorrhage and finally die. These phenomena occur both with active and inactive serum.

The immune body exerts its action when injected along with the spirochætæ and also when the spirochætæ are already in the tissues. When the serum of a convalescent patient is injected into a guinea pig which has already developed jaundice and hemorrhage, the spiro-

[9] This work was carried out by Drs. Hoki and Ito.

chætæ disappear from the blood after half an hour, and after $3\frac{1}{2}$ hours only a small number of the degenerative type of the organism can be detected in sections of the liver by Levaditi's method of staining. At the end of 8 hours no spirochætæ are seen, but in these instances we were able to find a few spirochætæ in the other organs, especially the kidneys and suprarenals, by using the silver impregnation method. Identical results were obtained with each of the six strains of spirochætæ that we now possess.

In order to determine the time of appearance of the immune substance, sixteen cases of Weil's disease were examined within the first 30 days of illness. The majority of the sera examined had been preserved for 2 years. Pfeiffer's reaction proved positive in one case on the 14th day, in two cases on the 15th day, in one case on the 22nd day, and in three cases between the 24th and 28th days of illness.

These results show clearly that the immune substance is fully formed about the 14th to the 15th day of the illness. On the other hand, examination of eight cases from the 4th to the 11th day all proved negative, while one case was negative on the 9th day and positive on the 20th day, and another was negative on the 7th day and positive on the 15th day of illness.

The immunity endures for a long period of time. Of thirteen cases examined later than a month since the illness, immune bodies were detected at the expiration of over $5\frac{1}{2}$ years (two cases), 3 years and 2 months (one case), 2 years and 4 months (two cases), and 1 year and 10 months (one case). This immune substance is specific in Weil's disease and is not present in the serum of healthy persons who have not had Weil's disease or in persons with jaundice of other nature.

Distribution of the Spirochætæ in the Human Body.[10]

Postmortem examinations were performed on twelve cases which died between the 9th and 16th days of illness. Tissues from eight cases were fixed in formalin solution, and tissues from one case which died on the 8th day of illness had been preserved in Orth's solution for 8 years. The examination showed that while the kidneys contain the spirochætæ in the largest number, yet in every case spirochætæ were detected.

[10] This work was done by Dr. Kaneko.

Spirochætæ in the kidneys were mostly within the uriniferous tubules, and the majority were contained in the substance of urinary cylinders (casts). However, a small number occurred within the detritus in the uriniferous tubules or was attached to the epithelial cells. In tissues from one case which succumbed on the 10th day localized accumulations of the spirochætæ in the act of invading the tubules were seen in the interstitial tissues. The spirochætæ in the urinary cylinders were most numerous in a case succumbing on the 9th day, and within the lumen of the uriniferous tubules in a case succumbing on the 14th day.

The liver showed spirochætæ in smaller numbers, of which many were degenerated. No spirochætæ were found in the specimen of liver preserved in Orth's solution. Within the liver the spirochætæ were chiefly present within the hepatic cells; they occurred frequently also at a comparatively early stage of the disease in the interstitial tissues and often also in the stellate cells of Kupffer. In cases dying about the 9th day the spirochætæ were typical in form and occurred within the veins and on the outer surface of the cells, while in cases from the 14th to the 16th day of illness they were present only within the hepatic cells. The distribution of the spirochætæ in the suprarenal gland is similar to that in the liver.

The lymph glands and spleen contained a small number of spirochætæ, mostly in a degenerated condition. They were chiefly inside of phagocytic cells, while comparatively well preserved organisms were seen in masses of coagulated blood. This condition of the lymph glands is to be distinguished from what occurs in locally infected glands; in the latter in six cases examined typical spirochætæ existed, although in small number, while in the autopsied cases typical organisms rarely were met with.

As regards the other organs, comparatively well preserved organisms were found in the cardiac muscle, voluntary muscle, testicle, and arterial walls of the autopsied cases. The cardiac muscle especially exhibited well preserved, typical spirochætæ within the cell bodies and in the interstitial tissues. The spirochætæ were detected at times in the lung, pancreas, intestines, gall-bladder, genitals, nervous system, and skin, but they were mostly degenerated.

Thus the distribution of the spirochætæ in the human body differs from that in the guinea pig in that the number present is smaller, the degenerative forms are more abundant, they are more within cells, the kidney contains more, and the liver and adrenal glands contain fewer; the cardiac muscles, voluntary muscles, and arterial wall contain typical organisms.

The above differences can probably be explained by the action of the immune body, which appears in the course of the disease. This immune body destroys and dissolves the spirochætæ, and consequently one finds a smaller number of them in man. The greater occurrence of intracellular organisms is probably due to the fact that the spirochætæ invade cells in order to escape from the action of the immune body; while the presence of the spirochætæ in the epithelial cells of the glandular diseases perhaps indicates its way of escape from the body. The fact that the number of spirochætæ found in the autopsied cases is small may be explained partly by imperfect preservation of tissues and mixed infection; and we believe, therefore, greater numbers will be detected by means of a perfected method of study in the early stages of cases of pure infection. This view has recently been supported by finding the liver in two cases, which died on the 6th day, as heavily infected with spirochætæ as in the guinea pig.

Prophylaxis.

The mode of infection of the disease and the excretion of the spirochætæ indicate the means and the necessity of prophylaxis. As already mentioned, the disease occurs frequently among coal miners who work in that part of a mine which is inundated with water got rid of by pumping. It is, therefore, necessary to remove the water and then to disinfect the ground, when it is found that the disease occurs among the workmen who work at certain definite places in the mines. Lime is effective for the disinfection of the ground. It is also necessary to avoid infection through the healthy skin and the alimentary canal. Thus, when the infected locality is large so that it cannot be disinfected thoroughly, recourse must be had to the method of active immunization. Our study of this question is still under way, so that results can only partially be mentioned here.

Guinea pigs were immunized with repeated injections of liver emulsion of the infected animal and later with a pure culture of the spirochætæ which had been killed by carbolic acid. The animals thus immunized did not develop the disease on the injection of the spirochætæ, which, it was known, would produce the disease in healthy animals. Hence this method seems promising for the prevention of the disease in man. Our conclusion is that the flea and mosquito have no share in the infection.

Moreover, it is necessary to disinfect the urine for at least 40 days from the beginning of the illness, in view of the fact that the spirochætæ are incessantly excreted in the urine during the course of the disease, and even as late as the 40th day. Although the spirochætæ appear to be in small numbers only in stools, they should be disinfected, as should also bloody sputum.

The spirochætæ seem to live in water and mud and to invade the human body from there; hence it is important to study the conditions necessary for their existence and especially the question of temperature, and to discover the existence of the spirochæta carrier.

Experimental Therapeutics.

Treatment with Salvarsan.—Guinea pigs were used for this purpose. The method and time of the inoculation with the spirochætæ were different in various cases. We could not discover the spirochæta in the liver under dark-field illumination in fourteen out of nineteen guinea pigs which underwent treatment with salvarsan (0.1 gm. of salvarsan per kilo of body weight), but found jaundice and hemorrhage in six instances after inoculation of an emulsion made from livers in which spirochætæ were not found.

Of twenty-one guinea pigs which were treated with salvarsan (0.05 gm. of salvarsan per kilo of body weight), dark-field illumination showed spirochætæ in nine instances, and inoculation from the nine cases gave positive results in seven instances.

Examination of the blood for spirochætæ before and after the salvarsan injection was done in seven animals. Three cases proved negative after the injection, although they showed spirochætæ in the blood before the injection. In one animal spirochætæ were absent before the injection and were present for a day after the injection.

The other three animals proved negative both before and after the injection. From these results it may be concluded that the spirochætæ are removed from the blood by salvarsan.

The action of salvarsan against the spirochætæ in the liver and other organs is still under investigation and will be reported later. The animals often died from 4 to 8 days after salvarsan injection. Professor Hata informs us that this is due to the fact that the resistance of the guinea pig against arsenical compounds is low. This weak resistance to arsenic is unfortunate, since the guinea pig is the only animal possessing strong susceptibilities for the spirochætæ.

Treatment with Immune Serum.—At first we used serum taken from a convalescent patient, and later serum from an immunized goat. The treatment with both sera gave better results than did the salvarsan treatment.

1 to 2 cc. of the serum from the convalescent patient were injected, before the appearance of jaundice, into six guinea pigs. Five of them developed no typical symptoms and no spirochætæ were found in the liver.

One of the animals developed hemorrhage and jaundice, but upon examination of the liver no spirochætæ were found. Six animals which received the serum injection, after the appearance of jaundice, showed postmortem typical changes, but spirochætæ were present in the liver in two instances only.

Immune goat serum was injected into twelve animals before the appearance of jaundice and at the time when the fever developed and spirochætæ were present in the blood. The spirochætæ disappeared from the blood half an hour after the serum injection and all the animals remained alive, except one which died on the 3rd day. This animal showed typical postmortem changes, but spirochætæ were not found in the liver, and the inoculation test with the liver emulsion also proved negative. Two of the remaining eleven animals developed slight jaundice, but recovered.

The injection of immune goat serum is ineffective after jaundice has appeared, and the guinea pig dies after the treatment. We did not find spirochætæ in the liver, and the inoculation test was negative, as was the microscopical examination of the organ. These results indicate a great difference between salvarsan and immune serum in treating the experimental disease.

The temperature of the animals which received the immune serum before the appearance of jaundice does not fall as in collapse, but falls at first and then rises again slightly. This phenomenon resembles that of the fever following the crisis in Weil's disease.

The strength of our immune goat serum is the same as that of the patient in the convalescent stage of the disease. Whether the strength can be increased has not yet been determined.

The serum treatment of the experimentally infected animals has, so far, given satisfactory results and affords a good basis for the clinical use of the serum treatment on man. The serum treatment is not suitable for cases which suffer relapses, but may be considered hopeful for cases which have already developed an immune body in the course of the disease, with lasting immunity.

We have also immunized horses for the purpose of obtaining an immune serum and we have had at least a partial success with horse serum. In a case in which it was administered subcutaneously the spirochætæ disappeared from the blood within 24 hours.

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EXPLANATION OF PLATES.

PLATE 56.

FIGS. 1 to 5. Microphotographs of *Spirochæta icterohæmorrhagiæ* in human blood. Giemsa stain. × 750.

FIG. 1. H. K. 6th day of illness.

FIG. 2. I. H. 4th day.

FIG. 3. I. Y. 11th day.

FIG. 4. I. Y. 11th day.

FIG. 5. Y. T. 8th day.

FIGS. 6 to 9. Spirochætæ in the blood of a guinea pig.

FIG. 6. Giemsa stain. × 750.

FIGS. 7 and 8. Silver impregnation. × 1,000.

PLATE 57.

FIG. 9. Two spirochætæ intertwined.

FIG. 10. Cover-glass specimen of the liver of a guinea pig. Giemsa stain. × 2,500.

FIG. 11. Spirochætæ in the liver of a guinea pig which developed the disease after an injection of *Spirochæta icterohæmorrhagiæ*.

FIG. 12. Spirochætæ intertwined.

FIG. 13. A degenerative type.

FIG. 14. Spirochætæ ending in a cluster.

PLATE 58.

FIG. 15. A spirochæta in the skin of a guinea pig 10 hours after infection. The spirochæta is in the corium.

FIGS. 16, 17, and 18. Spirochætæ excreted in the urine of patients.

FIG. 16. Numerous normal spirochætæ in a cylindroid from a patient (S.) on the 16th day of the disease. Dark-field illumination.

FIG. 17. Numerous degenerative types in a cylindroid from a patient (I.) on the 23rd day of the disease.

FIG. 18. Microphotograph of spirochætæ in the urinary sediment from the same patient as Fig. 16.

PLATE 59.

FIGS. 19, 20, 21, 22, and 23. Spirochætæ in the organs in spirochætosis icterohæmorrhagica.

FIG. 19. Degenerative type of spirochætæ in the liver (intracellular) of a patient autopsied on the 13th day of the disease.

FIG. 20. Spirochætæ in the kidney of a patient autopsied on the 10th day of the disease.

FIG. 21. Spirochætæ in the cylinder of the kidney of a patient autopsied on the 8th day.

FIG. 22. Spirochætæ in the liver of a patient autopsied on the 6th day. × 350.

PLATE 60.

FIG. 23. Drawing of Fig. 22.

FIG. 24. Microphotograph of spirochætæ in the uriniferous tubules of a guinea pig, which recovered after treatment with immune serum. Killed after 21 days.

PLATE 61.

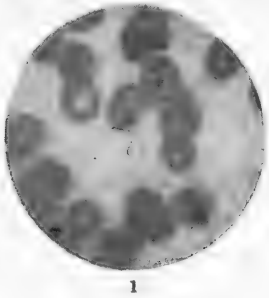
FIG. 25. Forms of *Spirochæta icterohæmorrhagiæ*.

1 to 65. Giemsa stain. 66 to 87. Silver-impregnated spirochætæ from tissues. 1 to 25 and 66 to 68. Various forms of the spirochæta. 26. A spirochæta 25 μ in length. 27 to 32, 73 to 74, and 77 to 87. Various forms of the degenerative type. 33 to 41, and 43. A mass of chromatin (?) and the so called bud of the spirochæta. 42, 69, and 70. A spirochæta ending in a cluster. 44 to 47, 75 and 76. The so called bud of a short spirochæta. 48 to 51. A spirochæta which resembles a platinum loop at one end. 52 to 59, 71 and 72. A spirochæta forming a ring. 60 to 63. A spirochæta with a small ring. 64 and 65. Forms which are considered to be in the stage of multiplication.

PLATE 62.

FIG. 26. Forms and movements of *Spirochæta icterohæmorrhagiæ*. The pure culture is observed under dark-field illumination.

1. Forward motion. 2 to 4. Rotating motion. 5. Rotating motion of a less active spirochæta. 6. A snake-like movement. 7 and 8. Young, short forms. 9. Grouping in the form of a rosette. 10 to 12. A group of spirochætæ. 13. Pair-form. 14 and 15. A mass of chromatin (?). 16. A spirochæta ending in a cluster. 17 and 18. Long forms (two and three times the length of the usual spirochætæ). 19. A straight form. 20. A thick form. 21 to 29. Various degenerative forms (crooked form). 30 to 34. Other degenerative forms. 35. A transition form between the above and the normal. 36. Spiral movement.



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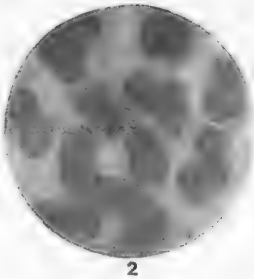

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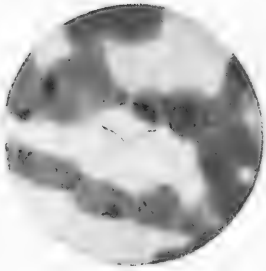

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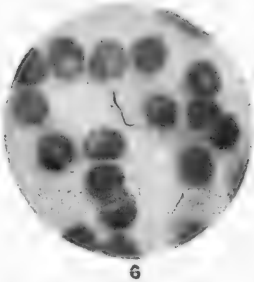

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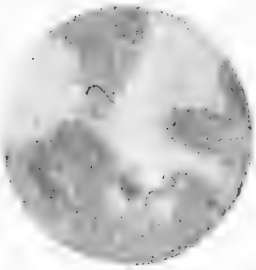

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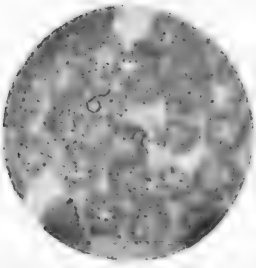

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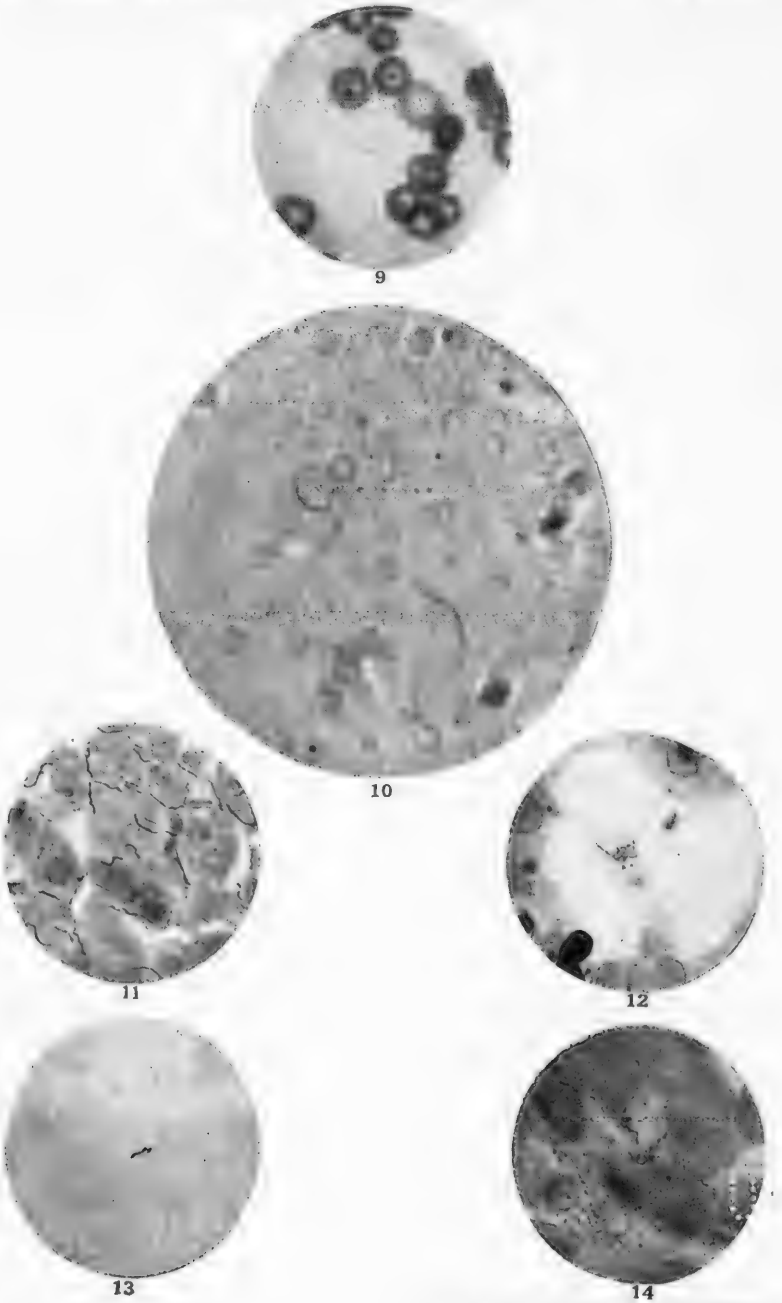
(Inada, Ido, Hoki, Kaneko and Ito: Weil's Disease.)



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(Inada, Ido, Hoki, Kaneko and Ito: Weil's Disease.)

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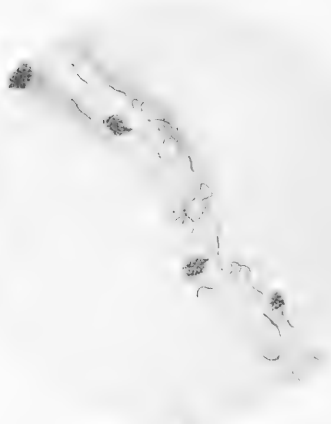

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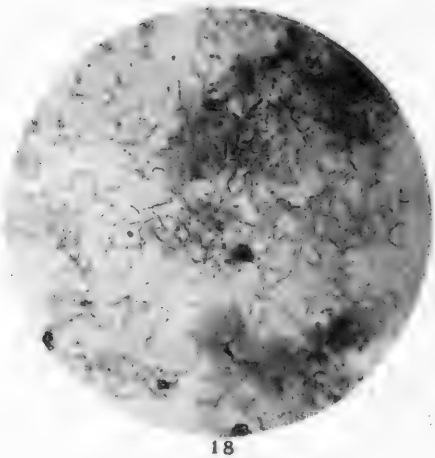

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(Inada, Ido, Hoki, Kaneko and Ito: Weil's Disease.)

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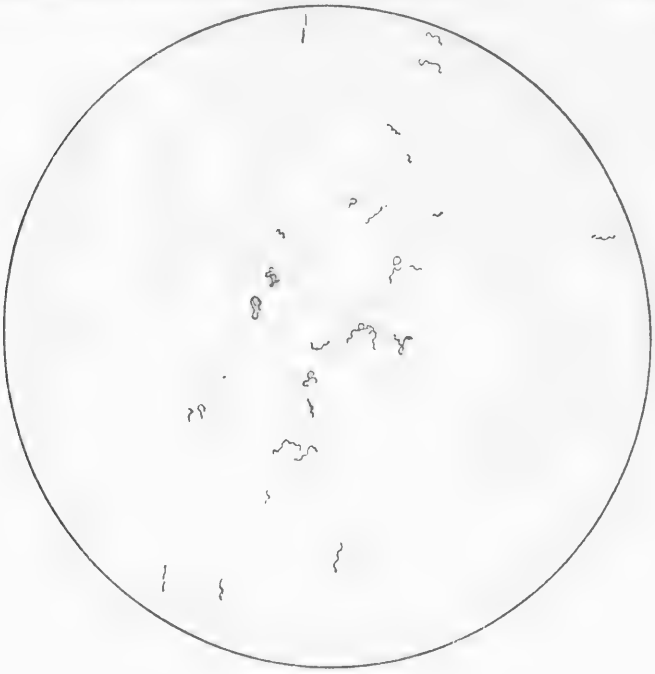
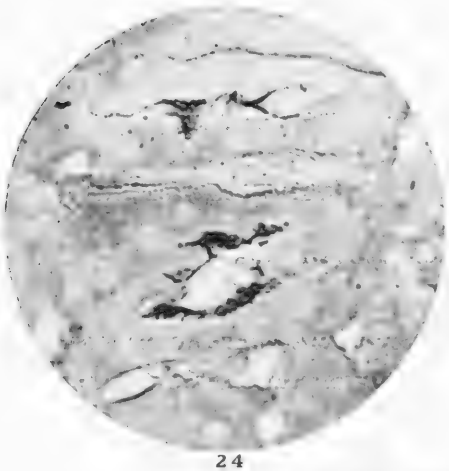


FIG. 23.



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(Inada, Ido, Hoki, Kaneko and Ito: Weil's Disease.)

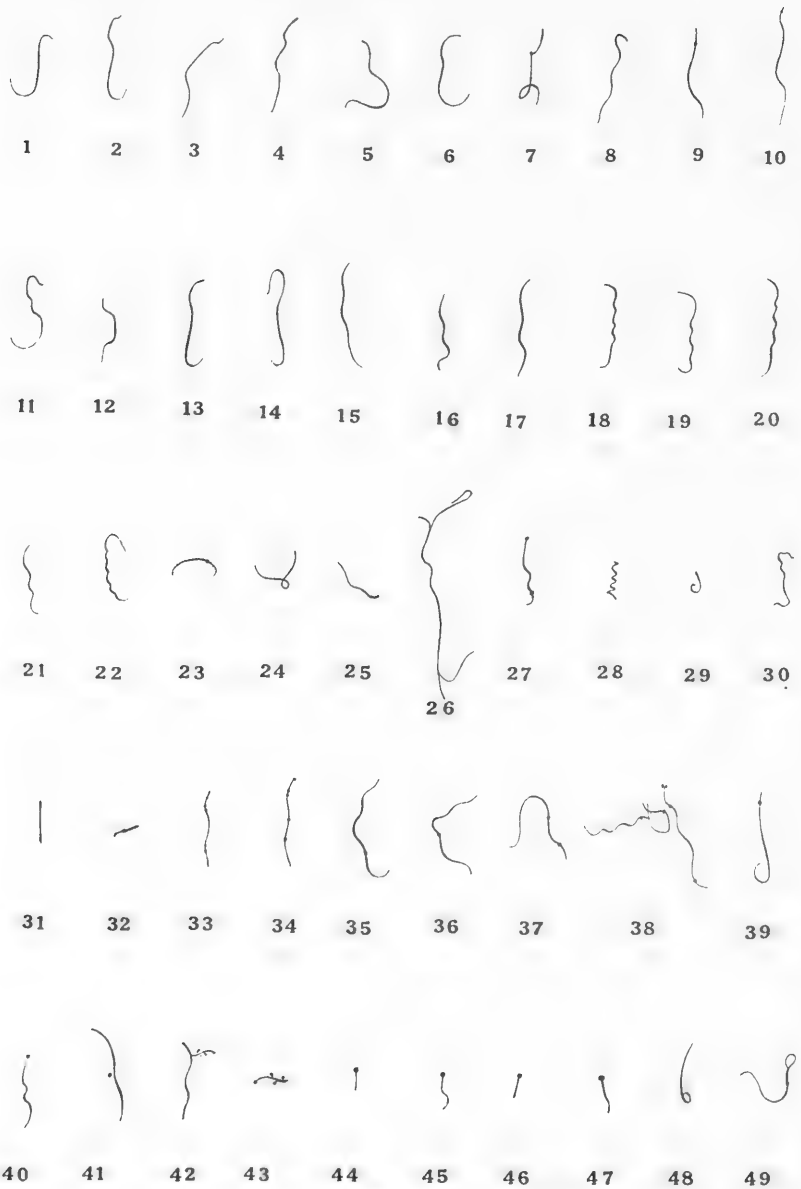
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FIG. 25a.

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FIG. 25b.
(Inada, Ido, Hoki, Kaneko and Ito: Weil's Disease.)

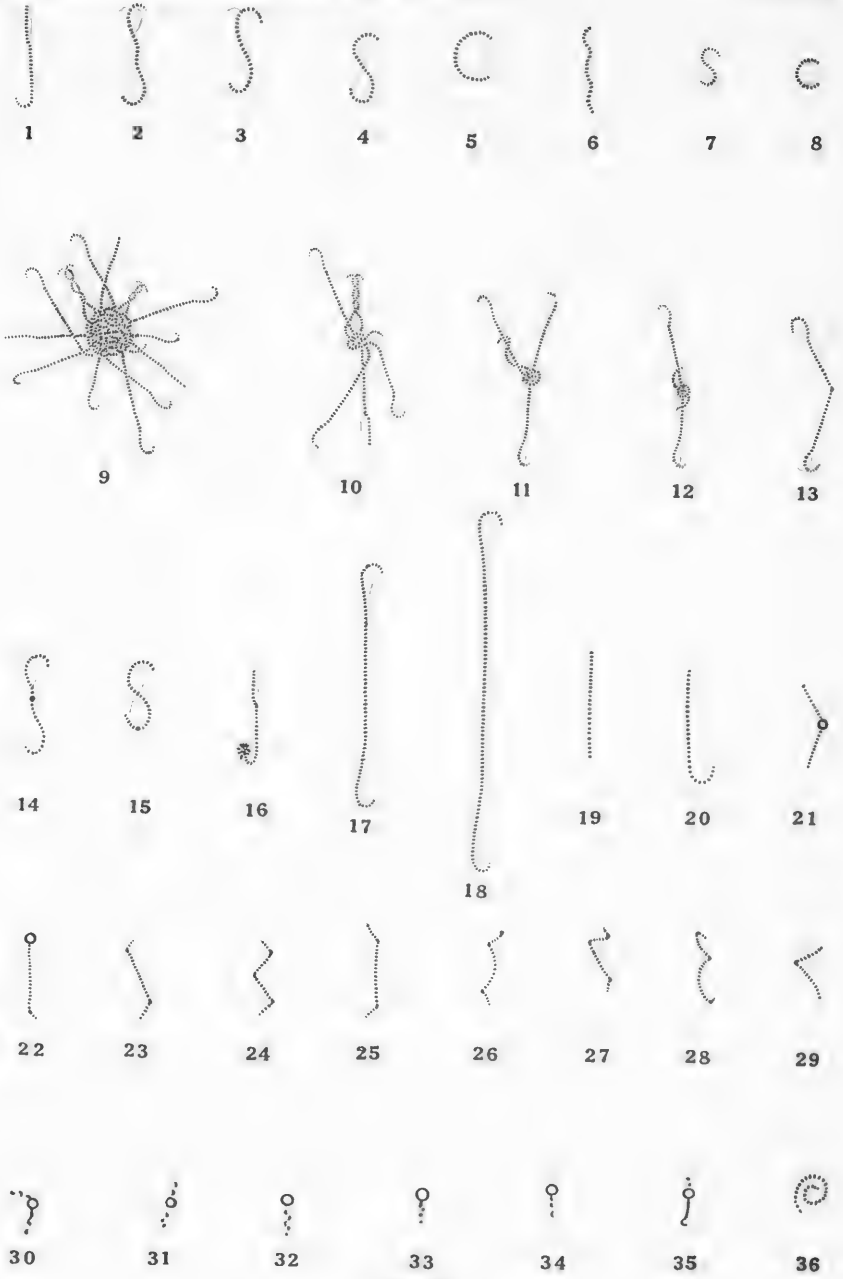

FIG. 26.

(Inada, Ido, Hoki, Kaneko and Ito: Weil's Disease.)

A METHOD FOR THE RAPID PREPARATION OF ANTI-MENINGITIS SERUM.

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The European war has brought about a greatly increased demand for antimeningococcic serum. Epidemic meningitis is known to be one of the attendants of armies in barracks and in the field, and the present war has proven no exception to this rule. Information is at hand indicating that at least the principal, if not all the belligerent countries have suffered from epidemics of meningitis to a greater or less extent. Now that we have knowledge of the manner in which epidemic meningitis is conveyed, namely, by means of meningococcus carriers who harbor that microorganism in the nasopharynx, the appearance of meningitis in widely separated countries and places is not at all remarkable. Meningococcic meningitis has prevailed either in epidemic form or sporadically in many European countries during the past 10 or more years. Hence the bringing together of recruits has had the effect of introducing carriers of the infection into the midst of the European armies. Not until the European war is over and the medical data of the war have been collected and analyzed shall we know whether the epidemics have been of equal severity throughout or whether some have been of greater severity than others. But the interim observations and studies have already yielded certain definite information of great importance in respect to the combating of the disease through the employment of the specific antimeningococcus serum.

Types of Meningococcus.

Biological studies of meningococci have led to their separation into two main groups, called respectively meningococci, or normal

meningococci,[1] and parameningococci (Dopter[2]). The two main groups are distinguished not by their cultural properties, but by their immunity reactions. This divergence in immunity reaction has proven of the greatest importance in perfecting the antimeningitis serum, since its action is specific and it affects those types of meningococci only for which its antibodies possess affinity.

At the outset, and before the two main groups of meningococci were clearly differentiated, the antimeningitis serum was prepared by employing a number of strains of meningococci in the immunizing process. The object, of course, was to cover ordinary variation in immunity properties of the different cultures; but with the discovery of the two types of meningococci this haphazard method no longer sufficed and it became necessary to use for purposes of immunization representatives of the two groups now designated meningococci and parameningococci. The sera now being prepared are for the most part produced by immunizing horses either with mixtures of these types of meningococci or with alternate injections of the two types.

Our recent studies indicate, however, that it does not suffice merely to employ representative normal and representative parameningococcus strains for the purpose of immunization, for while the two groups are, immunologically considered, fairly homogeneous, yet within each group there exist organisms which react weakly to the specific immune bodies produced by other strains of the same type. On the other hand, when these weakly reacting strains are themselves inoculated they evoke the formation of specific immune bodies to which they react strongly. Hence it is desirable to employ for immunization not merely one, but rather several strains of each group; and it is further desirable to change or alternate the strains as new ones are obtained which show an imperfect response to the immunity bodies already present.

The existence of different types of meningococcus was indicated early in the course of the serum treatment of meningitis through the observation that in certain cases the meningococci when brought under the influence of the antiserum failed to be affected by it. Since this was the exception, it was assumed that certain strains of meningo-

[1] Wollstein, M., *Jour. Exper. Med.*, 1914, xx, 201.

[2] Dopter, C., *Compt. rend. Soc. de biol.*, 1909, lxvii, 74.

cocci were resistant, or fast, to the antimeningitis serum. Further studies, as has been mentioned, established the fact that two great groups of meningococci could be distinguished; and our later studies indicated that even within these groups variants occur which are less subject to the action of a polyvalent antimeningitis serum than the majority of strains.

We are at present only imperfectly informed as to the relative prevalence of the different types of meningococci in given foci of epidemic meningitis. The American experiences and apparently the experiences previous to recent studies in the war zone in France and England have indicated that normal meningococci greatly preponderate in cases of epidemic meningitis. It now appears that, in certain localities at least in which epidemic meningitis prevails in France and England among the army, the proportion of cases caused by normal meningococci as contrasted with cases caused by parameningococci may be no higher than 6 : 4 (Ellis,[3] Arkwright[4]).

This consideration has definite bearing on the preparation of antimeningitis serum and emphasizes the importance of proceeding in its preparation in such a manner as to produce quantitative results in which the antibodies for the parameningococci about equal in amount those for the normal strains.

Mode of Preparation of the Serum.

At the first appearance of the present outbreak of epidemic meningitis in Great Britain, The Rockefeller Institute was no longer engaged in the preparation of the antimeningitis serum. Two circumstances led to the resumption of its manufacture. The first was the probability that the epidemics abroad would extend and the demand for the serum would exceed that available from ordinary sources of supply. Moreover, the identification of The Rockefeller Institute with the original production of the serum brought to it urgent requests for serum from several of the countries at war, with which it seemed imperative to comply. Through the assistance rendered by The Rockefeller Foundation, which has been engaged extensively in war

[3] Ellis, A. W. M., *Brit. Med. Jour.*, 1915, ii, 881.
[4] Arkwright, J. A., *Brit. Med. Jour.*, 1915, ii, 885.

relief, funds were placed at the disposal of the Institute covering the cost of production of the serum, so that it could be supplied gratis to those countries from which the demand came.

But there was a second important reason which led to the resumption of the manufacture of the serum. At the time and after the appearance of epidemic meningitis, particularly among the British recruits, the supply of serum available in England was chiefly that prepared commercially. Its use was distinctly disappointing. Realizing that in all probability the failure lay with the samples of serum available, which by reason of some fault of production or preservation were inactive, it seemed desirable to produce a serum the activity of which could be relied upon (Osler, Rolleston[5]).

In a previous communication[6] from the laboratories of the Institute a method was described by means of which the preparation of the antidysenteric serum was greatly abbreviated. The method consists in making injections into the horse of cultures or extracts of dysentery bacilli on 3 successive days, after which a period of rest is permitted. It was ascertained that the immunity response to the bacteria or bacterial products thus injected was far greater than when they were introduced at periods separated from each other by the ordinary intervals of time. Moreover, it was determined that by this rapid method an efficient polyvalent antidysenteric serum could be produced, representing both the non-acid (Shiga) and the acid (Flexner) groups of bacilli. Instead of a period of 8 or 12 months required by the usual method of immunization, an equally strong serum was produced in the short period of 8 or 12 weeks.

The problem presented, therefore, in respect to the antimeningitis serum seemed essentially similar to that encountered with respect to the antidysenteric serum. In the case of the latter, two groups of the bacilli are dealt with: first, a fixed or Shiga group, which yields a soluble toxin; and second, a fluctuating group made up of very slightly divergent types of bacilli which do not yield a soluble toxin. In the case of the meningococci there are also two groups or types, neither being perhaps altogether fixed: the ordinary or normal meningococci

[5] Discussion on the treatment of cerebrospinal meningitis, *Proc. Roy. Soc. Med., Therap. and Pharmacol. Sec.*, 1915, ix, 1.

[6] Flexner, S., and Amoss, H. L., *Jour. Exper. Med.*, 1915, xxi, 515.

which readily undergo autolysis, and the parameningococci undergoing less perfect autolysis, both yielding, however, a toxic product. Hence it could be assumed that by employing a method similar to that worked out for the antidysenteric serum, horses might be rendered immune and made to yield an efficient polyvalent antimeningococcic serum in a period of time far shorter than is required by the usual method of subcutaneous inoculation. The substances employed in both instances are the same; namely, living cultures and the extract, or autolysate, inoculated alternately. By the old method from 6 to 12 months were required to produce a meningococcic serum of high immunity value. By the new method a similar result has been achieved in from 8 to 12 weeks.

In following out this plan, a difficulty was early encountered, and one which was indeed foreseen. Two general methods of immunization of horses have been followed for the production of antimeningitis serum. In one the cultures of meningococci or the autolysate is injected subcutaneously (Flexner and Jobling[7]); in the other, the injections are made intravenously (Dopter[8]). In the first instance, no ill effects arise, aside from the occasional production of sterile abscesses. In the other, after several inoculations have been made, the horses become extremely sensitive, so that sudden death has been known to follow an injection of the culture or autolysate. This danger can, however, be prevented, as has been shown by Dopter,[9] by employing a desensitizing injection of the culture before making the full inoculation.

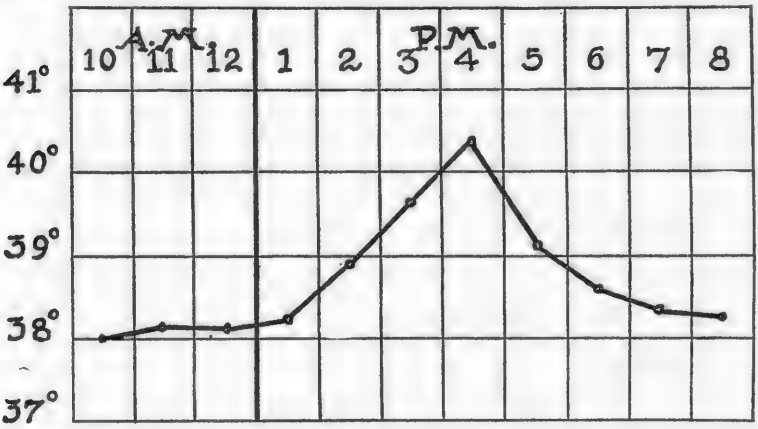
Hence the method adopted for the rapid production of the antimeningitis serum follows closely, but does not exactly reproduce, that already described for the rapid production of the antidysenteric serum. It consists in beginning with small doses of living meningococci injected daily for 3 days, followed by a period of rest of 7 days, when another series of injections is made. Thus for a 1,300 pound horse, one-twentieth of a 24 hour culture on slanted plain agar is injected on the 1st day. The material for injection is made up as follows: 2 cc. of physiological salt solution are added to a 24 hour agar slant

[7] Flexner, S., and Jobling, J. W., *Jour. Exper. Med.*, 1908, x, 141.

[8] Dopter, C., *Ann. de l'Inst. Pasteur*, 1910, xxiv, 96.

[9] Briot and Dopter, *Compt. rend. Soc. de biol.*, 1910, lxix, 174.

of meningococcus culture and the growth is suspended in it. Then 0.1 cc. of the suspension is transferred to 15 cc. of physiological salt solution and injected intravenously very slowly. The temperatures are taken hourly, beginning with the 4th hour after the injection, and continued until the temperature has reached its highest and begun to decline. 24 hours later 0.2 cc. of the suspension, and on the 3rd day 0.3 cc., that is about one-seventh of the agar slant, is used. After the lapse of 7 days, the dose given on the first of the 3 days corresponds with that given at the end of the last series; namely, in the instance in question, 0.3 cc. The temperature is again taken, and



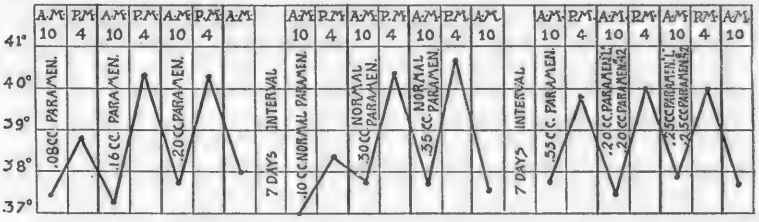
TEXT-FIG. 1. Typical febrile rise and fall following each single injection of meningococci.

if the rise does not equal 2.5–3°C. the conclusion drawn is that the dose has been too small. It is increased, therefore, for the injection 24 hours later above the usual rate of increase of 0.1 cc. according to the degree of rise of temperature. If the temperature does not fall to normal within 18 to 24 hours, the conclusion is drawn that the dose given has been too large (Text-fig. 1).

Following this plan, doses may be regulated with nicety and a maximum of reaction be obtained, we believe, with a minimum of danger. No serious effect is produced, although chills may attend the severer reactions. The greatest reaction, as a rule, is that pro-

duced by the first injection, whereas the succeeding injections on the 2nd and 3rd day tend to produce less severe reactions. Hence the increase between the second and the third injection may be larger than that between the first and second. In the second series of injections, the maximum doses have been from 0.4 to 0.45 cc., and in the third series from 0.5 to 0.6 cc. on the 3rd day. Since individual horses vary in their susceptibility, the first doses are small and the temperature curve forms the basis for adjusting the other doses (Text-fig. 2).

In Horse N only two series of injections were necessary before the typical and desired temperature curve was attained. The succeeding curves for any series agree closely with the third series in the chart.



TEXT-FIG. 2. Chart of Horse N showing febrile reactions and adjustment of doses in the first three series of injections.

In the succeeding series increase in the amounts injected is accomplished by the addition of new strains from time to time. For example, when the total dose is 0.6 cc. (*i.e.*, slightly more than one-third of the agar slant) it may consist of 0.2 cc. each of three strains. The largest amount of any single injection in a 1,300 pound horse has been one-fourth of each slant from seventeen different strains.

In preparing a polyvalent serum two slightly different procedures may be followed. According to one, the normal and the parameningococcus strains are inoculated within what may be designated as periods of two series; that is, the normal and the parameningococcus strains are alternated. According to the other, in which the autolysate is also employed, the period includes three series, one for the normal meningococcus, one for the parameningococcus strains, and one for the autolysate. The autolysate, in turn, is made up of equal parts

of a typical normal meningococcus and of a typical parameningococcus strain. Evidence is at hand, to be referred to below, which shows that the autolysate, at least in the sheep, excites little agglutinin formation while producing other protective principles.

Desensitization.

As stated, the horse becomes hypersensitive to the intravenous injections of the meningococci or its products apparently after the third or fourth series of injections. This reaction tends to be most severe after the first dose in each series, as might have been predicted. It was found that, acting upon the suggestion of Dopter,[9] desensitization may be effected apparently readily and certainly. Hence on the 1st day of each series about one-twentieth (later one-tenth) of a 24 hour slant of culture is injected intravenously, and 2 hours later the remainder of the dose is given. This desensitization suffices and need not be repeated on the 2nd and 3rd days, when the next doses are injected.

The danger from both brain and lung emboli must not be overlooked. Bull[10] has observed that suspensions of bacteria, introduced into the blood stream of an animal immune to the same organism, are clumped and deposited in the blood vessels of the brain, lungs, and in the spleen. If the bacteria are present in any considerable number, the clumps may occlude the cerebral and pulmonary capillaries and produce sudden death of the animal. We have found this to be true in the injection of meningococcus into normal and immune rabbits. It is possible that a part of the so called anaphylactic phenomena observed in immunization with meningococci can be explained by these facts, since the symptoms observed are similar if not identical in these two instances.

In order to avoid such a possible danger the suspensions of living meningococci are made up to a relatively larger volume, 15 to 20 cc., and introduced very slowly into the circulation.

[10] Bull, C. G., *Jour. Exper. Med.*, 1915, xxii, 466.

Strains Employed in the Immunizing Process.

In the beginning a group of five representative normal meningococcus strains and a group consisting of an equal number of parameningococcus strains were used alternately in the series of injections. The progress of the formation of immune bodies in the serum was followed by studying the agglutination, opsonization, and complement deviation reactions, with as many strains of meningococci as possible. In this manner strains not employed in the original series of inoculations may be divided into temporary lots according to their reaction with the serum. If the reactions are not within or near the zone of reaction of the strains already being used for immunization, representatives of such lots are selected and placed in the immunizing group. The remaining members of these lots are again tested later when immune bodies against the representative of the group have appeared in the serum. Should the serum now contain antibodies effective against all members of the group, it will, of course, not be necessary to include them all. Agglutination serves as the most specific index of immunological reaction except in the case of inagglutinable strains, which, in our experience, are infrequently encountered.

By the above procedure we have studied sixty-four strains of meningococci, and from this number twenty-two were selected to be added to the two original immunizing groups which consisted respectively of five normal meningococcus strains and five parameningococcus strains. For convenience in regulating the doses and for injecting we have added equally to each group.

We selected for addition to the normal group eleven of those which showed the least variation from the normal meningococcus; and the remaining eleven, exhibiting wider variations, were placed in the second group which originally consisted only of parameningococci. The strains used at present for immunizing are thirty-two, and have for convenience been divided into two lots. Lot A consisted originally of five normal and irregular strains of meningococcus, and to these have been added eleven normal and irregular strains. Lot B consisted originally of five parameningococcus strains (two from the Pasteur Institute, Paris, and three isolated in America) and to these have been added eleven parameningococcus and irregular strains exhibiting wider variation from the normal. One lot is used for three

successive injections and after a rest of 7 days the other lot is used in like manner. If care is not taken to balance the two lots, the immune bodies against one group may be developed to a greater extent than those against the other. To avoid this, the serum is tested immunologically after every second series if living organisms are injected, and after every third series if autolysate is also given. The relative doses of the two groups are regulated accordingly.

Since we have evidence that both normal meningococci and parameningococci differ among themselves in antigenic power, it is desirable to study all possible strains in the course of an epidemic in order that strains which are at variance with those being used in the production of an immune serum may be included in the immunizing groups.

This point is illustrated by the following experience with two strains. Strain NO was tested against the polyvalent serum of a horse highly immune against at least twenty normal and parameningococcus strains and found to be agglutinated in a dilution of 1 : 10. This strain, which was later found to be parameningococcus, was included in the immunizing group, and after two inoculations the agglutinins rose to 1 : 100, and after four injections to 1 : 200. When fewer strains are being used, the immune bodies may be developed more quickly. Strain Andrews, brought from England by Dr. A. Gardner Robb, when tested against a potent serum effective against ten normal meningococcus and parameningococcus strains, agglutinated in a dilution of 1 : 10. It was included in the immunizing group and after two series of injections, the agglutinating power of the serum for this strain rose to 1 : 200. We consider that the larger doses of Andrews which it was possible to employ caused the more rapid rate in the production of the agglutinins. Table I illustrates the development of agglutinins for strains relatively inagglutinable before their inclusion in the inoculation group.

TABLE I.

Strains.	Before injection.	After 1 injection.	After 2 injections.	After 4 injections.	After 5 injections.
Andrews.....	1 : 10	1 : 100	1 : 200	.....	1 : 2,000
NO.....	1 : 10	.....	1 : 100	1 : 200	.....

Autolysate.

Equal parts of toluene autolysate[11] from normal meningococcus and from parameningococcus may be injected intravenously on 3 successive days forming one series, alternating with the two series of living meningococci. The serum of horses receiving the autolysate series develops agglutinins, opsonins, or power to deviate complement less quickly than that of animals receiving only the living organisms, though power to neutralize the toxin contained in the autolysate is developed.

TABLE II.

Serum.	Agglutinins.		Opsonins.		Complement deviation.	
	Normal meningococcus.	Parameningococcus.	Normal meningococcus.	Parameningococcus.	Antigen from normal meningococcus.	Antigen from parameningococcus.
Sheep B (autolysate).....	1 : 100	1 : 50	1 : 50	1 : 1,000	1 : 200	1 : 100
Horse L (living cultures and autolysate).....	1 : 5,000	1 : 2,000	1 : 2,000	1 : 5,000	1 : 10,000	1 : 5,000

A sheep received intravenously maximum doses of autolysate in series of 3 successive days and a rest of 7 days over a period of 9 months. Doses sufficient in size to cause marked febrile reaction and sometimes diarrhea, were administered. At the end of 9 months the serum exhibited only slight power of agglutination, opsonization, and complement deviation.

Table II shows the titer of the sheep serum compared with that of serum obtained from a horse receiving autolysate and also living meningococci.

The sheep serum when incubated with living meningococci and injected intraperitoneally into small guinea pigs was found to possess low anti-infectious value.

[11] Flexner, S., *Jour. Exper. Med.*, 1907, xx, 105.

Immunity Value of the Antiserum.

The immune bodies of the horse serum were estimated by testing its agglutinating and opsonizing power with normal and parameningococcus strains and by determining its power to fix complement in the presence of antigens made from these strains. Its anti-infectious power was determined by incubating varying amounts with one minimum lethal dose of living meningococci for 1 hour at 37°C., and injecting the mixture intraperitoneally into young guinea pigs weighing not less than 90 or more than 110 gm.

Agglutination.—Representative normal meningococci and parameningococci were selected for following the development of agglutinins.

TABLE III.

Horse L Serum.

Period.	Before injection.	1st.	2nd.	4th.	7th.*	After 6 wks. of autolysate injections.†	10th.	11th.‡	13th.	14th.
Meningococcus.....	1:30	1:60	1:80	1 : 1,000	1 : 5,000	1 : 2,000	1 : 2,000	1 : 500	1 : 1,000	1 : 2,000
Parameningococcus..	1:10	....	1:80	1 : 200	1 : 2,000	1 : 500	1 : 1,000	1 : 1,000	1 : 1,000	1 : 2,000

* 7 periods extend over 10 weeks. 6 liters of blood were taken from the horse at this time.

† For 6 weeks after the 9th period the horse received autolysate only.

‡ Twelve new strains added. 1st period after bleeding.

Horse M Serum.

Period.	Before injection.	2nd.	4th.	7th.	10th.
Meningococcus.....	1 : 20	1 : 500	1 : 500	1 : 1,000	1 : 2,000
Parameningococcus.....	1 : 10	1 : 50*	1 : 1,000	1 : 1,000	1 : 2,000

10 periods extend over 12½ weeks.

* This low figure shows that the injections had not been properly balanced. Too few parameningococci had been injected; accordingly larger doses were given with the result that the agglutinins increased greatly during the next periods. The highest dilution at which any of our sera agglutinated was 1 : 5,000 for both normal and parameningococci.

The reactions were made at 55°C. and read after 24 hours. Table III shows the increase of agglutinins by periods.

Opsonins.—The opsonins were estimated by the Neufeld technique. Table IV shows their development by periods.

TABLE IV.

Horse L Serum.

Period.	5th.	7th.	9th.	Interval of 6 wks. Injected with autolysate only.	10th.	13th.	15th.
Meningococcus.	1 : 500	1 : 1,000	1 : 500*		1 : 500	1 : 1,000	1 : 2,000
Parameningococcus.....	1 : 200	1 : 5,000	1 : 2,000		1 : 1,000	1 : 1,000	1 : 2,000

* Bled 6 liters just before this period.

Horse M Serum.

Period.	2nd.	7th.	11th.
Meningococcus.....	1 : 500	1 : 200	1 : 2,000
Parameningococcus.....	1 : 200	1 : 1,000	1 : 2,000

Complement Fixation.—Tests for complement-binding bodies in the immune horse and sheep sera were made with antigens of regular normal meningococci as well as with those of irregular normal meningococcus and parameningococcus strains. The results are shown in Table V. It appears that in the serum from Horse L the power to bind complement ran fairly parallel with the power to agglutinate the meningococci.

TABLE V.

Complement Deviation by Serum L.

Period.	4th.	7th.	8th.	9th.	Interval of 6 wks. Injected with autolysate only.	10th.	11th.	12th.	13th.	14th.
Meningococcus....	1:200	1 : 1,000	1 : 1,000	1 : 2,000		1 : 1,000	1:500	1 : 1,000	1 : 1,000	1 : 1,000
Parameningococcus..	1:100	1 : 500	1 : 1,000	1 : 2,000		1 : 500	1:500	1 : 500	1 : 2,000	1 : 5,000

Complement Deviation by Serum M.

Period.	2nd.	4th.	5th.	6th.	9th.	12th.
Meningococcus	1 : 200	1 : 2,000	1 : 5,000	1 : 2,000	1 : 2,000	1 : 1,000
Parameningococcus....	1 : 200	1 : 1,000	1 : 2,000	1 : 1,000	1 : 1,000	1 : 5,000

Protective Value.—Flexner recommended the use of small guinea pigs in determining the anti-infectious power of antimeningitis serum. If young guinea pigs weighing between 90 and 110 gm. are used and the experiment is run in quadruplicate, some measure of the protective power is obtained. One minimum lethal dose of the living meningococcus is incubated with varying amounts of immune serum for 1 hour at 37°C. and then injected intraperitoneally.

Table VI shows the anti-infectious power of serum L compared with normal horse serum.

TABLE VI.

Anti-Infectious Power of Polyvalent Antimeningitis Serum.

Strain.	2 cc. of normal horse serum.	0.2 cc. of polyvalent immune serum.	0.3 cc. of polyvalent immune serum.	0.4 cc. of polyvalent immune serum.
1 m. l. d. of living meningococci.	No protection.	Protection in 1 out of 4.	Protection in 3 out of 4.	Protection in 4.
1 m. l. d. of living parameningococci.	No protection.	Protection in 3 out of 4.	Protection in 3 out of 4.	Protection in 4.

If two minimum lethal doses are used, there is little protection after 48 hours. For example, in one series the control guinea pig receiving one minimum lethal dose and those receiving two minimum lethal doses plus 0.5 cc. of serum died in 12 to 18 hours. Three out of four guinea pigs receiving two minimum lethal doses plus 0.6 cc. of serum lived between 47 and 48 hours.

Normal horse serum exerts practically no anti-infectious action either with meningococcus or with parameningococcus. Immune serum produced by the rapid method possesses a considerable degree

of anti-infectious power. About 0.4 cc. of this polyvalent serum is capable of neutralizing the infecting power of one minimum lethal dose of the living meningococcus or parameningococcus.

SUMMARY.

Potent antimeningitis serum can be safely produced in the horse by the method of three successive intravenous inoculations of living meningococci and parameningococci repeated at stated intervals.

Sudden and alarming symptoms and sudden death are avoided by employing first a desensitizing injection and then by adjusting the doses according to the febrile reaction and by making the highly diluted injections slowly.

Horses undergoing this process of immunization remain in good condition and may even gain in weight.

Specific immune bodies appear in the serum early and rise rapidly.

By inoculating alternately several strains of living meningococci and parameningococci, and the autolyzed products of each, a polyvalent serum of high titer can be produced in 8 to 12 weeks instead of in the 10 months required by the subcutaneous method.

The serum produced by this rapid method has been employed therapeutically in America, England, France, and some other countries.

It is highly desirable to isolate meningococci from many sources and test the strains against the polyvalent serum. Strains which are not agglutinated in high dilution in such a serum should be included subsequently in the lot of strains used for immunization.

THE INFLUENCE OF TYPHOID BACILLI ON THE ANTIBODIES OF NORMAL AND IMMUNE RABBITS.

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The view is held that bacterial injections cause a decrease in the antibody content of the blood of man and the lower animals.[1] This belief is largely based on the work of Wright in connection with various forms of bacterial inoculation. Wright[2] contends that every process of immunization produces a sequence of negative and positive phases, and hence that repeated inoculation may give rise to a multiple of such sequences. This phenomenon is of particular interest in connection with prophylactic typhoid vaccination and in the treatment of cases of typhoid fever with intravenous injection of typhoid bacilli or their products.

In the course of a study of native and acquired immunity of rabbits to typhoid bacilli, including the fate of the bacilli when injected intravenously,[3] certain observations were made which threw doubt on the action of the negative phase as expressed above. These incidental observations have now been extended and the complete results are reported in this paper.

Technique.

Immunization of Animals.—Rabbits were immunized to typhoid bacilli as follows: 1st day, 0.1 of an agar slant of heat-killed bacilli was injected into the peritoneum; 2nd day, 0.1 of an agar slant of heat-killed bacilli was injected into the vein; 5th day, 0.1 of an agar slant

[1] von Wassermann, A., and Sommerfeld, P., *Med. Klin.*, 1915, xi, 1307.

[2] Wright, A. E., *Brit. Med. Jour.*, 1903, i, 1069.

[3] Bull, C. G., *Jour. Exper. Med.*, 1915, xxii, 475.

of living bacilli was injected into the vein; 7th and 9th days, repetition of the 5th day treatment. 10 days after the last injection the sera gave agglutination reactions in dilutions varying from 1 : 30,000 to 1 : 200,000.

Antibodies.—The immune sera were tested for agglutinins, precipitins, opsonins, complement fixation, and bactericidins. Every reaction was not, however, carried out with the serum of each rabbit, but different groups of rabbits were often used in the different sets of experiments, as shown in the individual tables.

Agglutinins.—The fresh sera were diluted with 0.9 per cent sodium chloride solution, ranging in strength from 1 in 2 to 1 in 200,000, as the individual animals demanded, and one drop of a suspension of typhoid bacilli washed from a 24 hour agar slant (growth covering the entire surface of the slant) with 6 cc. of salt solution, was added to 1 cc. of each dilution. The quantity of bacilli was kept as uniform as possible in the various tests of the same animal's serum, since it is known that the titer of a serum depends largely upon the number of bacilli present.[4] The tubes were incubated at 37°C. for 2 hours and allowed to remain at room temperature for 2 hours before the final readings were made.

Complement Fixation.—Antigen was prepared as follows: Typhoid bacilli were grown on plain agar, washed from the agar with salt solution, and freed from particles of the medium by repeated centrifugation. The washed bacillary bodies were frozen and thawed several times, dried in vacuum over sulphuric acid, and ground to a fine powder in an agate mortar. 1 dg. of the powder was added to 100 cc. of salt solution, after which the mixture was shaken for several hours and then passed through a Berkefeld filter. The clear filtrate was used as antigen in both the complement fixation and precipitin tests. It proved highly satisfactory. From 0.05 to 0.1 cc. gave binding with 0.04 cc. of immune serum, while 0.3 cc. did not bind with normal serum. A hen-rabbit hemolytic system and guinea pig complement were employed.

Precipitins.—In testing for precipitins 0.2 cc. of the antigen was floated over 0.2 cc. of the undiluted sera and the results were read

[4] Foerster, O., *Ztschr. f. Hyg. u. Infectionskrankh.*, 1897, xxiv, 500.

after standing for 2 hours at 37°C. The appearance of a delicate white ring at the plane of contact of serum and antigen was considered a positive reaction. The reaction was delicate and strictly specific. Filtrates from bouillon cultures of the bacteria gave nonspecific reactions.

Opsonins.—The opsonizing power of the sera was determined by the Neufeld method. Equal amounts of serum dilutions, bacterial suspension, and guinea pig leukocytes were mixed and incubated for 2 hours at 37°C.; slides were then made and stained after fixation in methyl alcohol with Manson's stain. The highest dilution of serum in which the degree of phagocytosis exceeded the salt solution control was considered as indicating the opsonic titer of the serum. This test was used more extensively in examining the normal than the immune sera. As typhoid bacilli are phagocyted in salt solution, the reactions are less delicate than the other tests employed.

Bactericidins.—The bactericidal capacity of the sera for typhoid bacilli was determined as described below.[5] Bacterial suspensions of varying strengths were prepared with sterile salt solution from a 24 hour bouillon culture. The suspensions ranged from 1 in 10 to 1 in 1,000,000. 0.05 cc. of the different suspensions was put in small test-tubes, and 0.2 cc. of the serum to be tested was added to each tube. Care was taken to prevent the bacterial suspensions from touching the sides of the tubes above the level of the sera and thus to escape the action of the serum. The tubes were incubated for 1 hour, and 1 cc. of melted agar was added to each tube, and also to a set of salt solution controls. After incubation the degree of destruction can be easily determined. Sterility of the tubes or a reduction in the number of colonies to three or four was regarded as a positive reaction. A mere reduction of colonies is no doubt to be ascribed to agglutination. This test was used especially for the sera of normal rabbits and for rabbits after the first inoculation of bacilli.

[5] Wright, A. E., *Lancet*, 1901, i, 609.

EXPERIMENTAL.

Each type of experiment reported has been performed a number of times, but only a few tables illustrating the different classes will be given in detail.

In Experiment 1 (Table I) three rabbits having a high degree of immunity to typhoid bacilli were used. A sample of blood was taken from each rabbit, injections and bleedings were made, and the sera tested, as indicated in Protocols A, B, and C (Table I).

TABLE I.

Rabbit A.

Injection.	⅛ of an agar tube of typhoid bacilli.				
Time of bleeding.	Before injection.	1 hr.	5 hrs.	24 hrs.	48 hrs.
Agglutinins	50,000	50,000	50,000	50,000	50,000
Precipitins	+++	+++	+++	+++	+++
Complement fixation	+++	+++	+++	+++	+++

Rabbit B.

Injection.	⅛ of an agar tube of typhoid bacilli.						
Time of bleeding.	Before injection.	1 hr.	5 hrs.	22 hrs.	47 hrs.	70 hrs.	120 hrs.
Agglutinins	80,000	80,000	80,000	80,000	80,000	80,000	100,000
Precipitins	+++	+++	+++	+++	+++	+++	+++
Complement fixation	+++	+++	+++	+++	+++	+++	+++

Rabbit C.

Injection.	⅛ of an agar tube of typhoid bacilli.				
Time of bleeding.	Before injection.	1 hr.	5 hrs.	24 hrs.	48 hrs.
Agglutinins	30,000	30,000	30,000	30,000	30,000
Precipitins	+++	+++	+++	+++	+++
Complement fixation	+++	+++	+++	+++	+++

Experiment 1 shows definitely that an intravenous inoculation of typhoid bacilli causes no reduction in the concentration of agglutinins,

precipitins, and degree of complement fixation of the sera of rabbits up to time limits of the tests. This result was surprising. It was to be expected in view of Wright's results that an injection of the homologous bacteria would cause lowering of the various antibodies. No such reduction was detected.

The results of this experiment led to the performance of a large number of experiments of a similar nature, the number of bacilli inoculated being varied widely. It was found that the number of bacilli injected did not influence the end result. Injections were given under the skin and into the peritoneum with like results. Intravenous inoculation of the bacilli from an entire agar slant caused no reduction in the concentration of the antibodies. Lethal doses of bacilli were given and the rabbits bled while dying, but still the sera contained agglutinins, precipitins, and complement deviating antibodies in the same concentration present before the injections were made. Moreover, when sublethal doses were employed, the agglutinins were found to be increased at the 48th to the 96th hour after inoculation.

Natural and acquired antibodies show some points of difference: opsonins are thermolabile (Wright), bacteriotropins thermostabile (Neufeld); the natural agglutinins of rabbits for typhoid bacilli are thermolabile and disappear spontaneously within a few days, while the acquired agglutinins are thermostabile and persistent. Hence, it was thought possible that the bacillary injection might cause a reduction in the natural antibodies, while the acquired ones might remain uninfluenced.

Rabbits can be used advantageously for these experiments, since the sera of a large percentage contain natural opsonins, agglutinins, and bactericidal substances for typhoid bacilli. Representative protocols of experiments pertaining to the natural antibodies follow (Table II).

The protocols of Experiment 2 (Table II) represent three types of test. In one the number of bacilli injected was sufficient to cause acute intoxication and death in from 1 to 3 hours. Rabbits A^1 and B^1 fall in this group. From 1 to 2 hours after the injections the rabbits were in a state of collapse; they showed extreme relaxation, low blood pressure, heavy breathing, and, as a rule, diarrhea. Specimens of blood were taken while the rabbits were in this state or immediately

TABLE II.

Rabbit A[1].

Date.	Time of bleeding.	Injection.	Opsonins.	Agglutinins.	Remarks.
1915					
Nov. 17	11.35 a.m.		1–3+	1–15+	
	11.40 a.m.	$\frac{1}{20}$ agar slant of typhoid bacilli in vein.			
	1.15 p.m.		1–3+	1–20+++	Rabbit moribund when bled and died immediately afterwards.

Rabbit B[1].

Date.	Time of bleeding.	Injection.	Opsonins.	Agglutinins.	Remarks.
1915					
Nov. 17	11.25 a.m.		1–6+	1–15+	
	11.30 a.m.	Same as Rabbit A[1].			
	1 p.m.		1–6+	1–20+++	Blood taken from heart immediately after death.

Rabbit C[1].

Date.	Time of bleeding.	Injection.	Opsonins.	Agglutinins.	Remarks.
1915					
Nov. 19	10 a.m.		1–20+	1–40+	
" 19	12 m.	$\frac{1}{20}$ agar slant of typhoid bacilli in vein.			
" 19	2 p.m.		1–20+	1–40++	Heavy breathing; collapse; diarrhea.
" 19	5 p.m.		1–20+	1–40+++	Rabbit very weak.
" 20	10 a.m.		1–20+	1–90+	
" 21	9.30 a.m.		1–24++	1–90+	
" 22	8 a.m.		1–50+	1–300+	Rabbit found dead, and unclotted blood removed from heart. Blood sterile.

TABLE II—*Continued.*

Rabbit D[1].

Date.	Time of bleeding.	Injection.	Opsonins.	Agglutinins.	Remarks.
1915					
Nov. 19	10 a.m.		1–12+	1–40+	
" 19	12 m.	Same as Rabbit C[1].			
" 19	2 p.m.		1–12+	1–40+	Collapse; diarrhea.
" 19	5 p.m.		1–12+++	1–60+	
" 20	9 a.m.		1–12+++	1–60+	
" 21	10 a.m.		1–16+++	1–80+	
" 21	11.45 a.m.	Rabbit died. Two agar slants and one tube of bouillon were each inoculated with one loop of heart's blood. One agar tube had two colonies, one was sterile; the bouillon gave a culture.			

Rabbit E[1].

Date.	Time of bleeding.	Injection.	Opsonins.	Agglutinins.	Remarks.
1915					
Nov. 19	10 a.m.		1–12+	1–30+	
" 19	10.20 a.m.	$\frac{1}{10}$ agar slant of typhoid bacilli in vein.			
" 19	12.20 p.m.		1–12+	1–30+	Rabbit flaccid.
" 19	3.20 p.m.		1–12+	1–40+	
" 20	10 a.m.		1–12+	1–40+	
" 21	9 a.m.		1–12+	1–50+	
" 22	10 a.m.		1–24+	1–70+	
" 23	10 a.m.		1–160+	1–200+	
" 24	10 a.m.		1–160++	1–500+	
" 27	12 m.		1–300+	1–5,000+	
" 27	12.30 p.m.	$\frac{1}{20}$ agar slant of typhoid bacilli in vein.			
" 27	2.30 p.m.		1–300+	1–5,000+	
" 27	6 p.m.		1–300+	1–5,000+	
" 28	9 a.m.		1–300+	1–5,000++	
" 29	9 a.m.		1–400+	1–20,000+	Rabbit in good condition.

TABLE II—*Concluded.*

Rabbit F[1].

Date.	Time of bleeding.	Injection.	Opsonins.	Agglutinins.	Remarks.
1915					
Nov. 19	2 p.m.		1–12+++	1–40+	
	2.45 p.m.	$\frac{1}{10}$ agar slant of typhoid bacilli in vein.			
" 19	5 p.m.		1–16+	1–60+	
" 20	8.30 a.m.		1–12+	1–60+	
" 21	9 a.m.		1–20+	1–120+	
" 22	9 a.m.		1–40++	1–280+	
" 22	10.15 a.m.	$\frac{1}{10}$ agar slant of typhoid bacilli in vein.			
" 22	12.15 p.m.		1–40++	1–280+	
" 22	3.15 p.m.		1–40++	1–280+	
" 24	9 a.m.		1–60+++++	1–400+	Rabbit in good condition.

after death and compared for opsonins and agglutinins with the blood taken before the injection. The sera obtained subsequent to the administration of the bacilli agglutinated the bacilli more actively and in higher dilutions than those taken before the bacilli were injected. A few exceptions were noted, but a lowering of the agglutinating strength never occurred. An increase in opsonizing power was not observed; no difference could be detected between the sera in this respect before and after inoculation. This fact is probably due to the circumstance that the opsonic reaction is less delicate than the agglutination test.

In another series, represented by Rabbits C[1] and D[1], fewer bacilli were injected and the rabbits succumbed in from 1 to 3 days to a more chronic intoxication. The chief symptoms noted were emaciation, anemia, and loss of appetite. At autopsy, the blood was sterile or contained very few bacilli. The sera frequently showed an initial increase in agglutinating power a few hours after the inoculation, and then a steady increase in from 24 to 48 hours and continuing up to the time of death.

In the case of Rabbit C^1, the serum obtained before the bacilli were given showed agglutination in a dilution of 1 in 40, while at about the 72nd hour the reaction was present in a dilution of 1 : 300. This rabbit was markedly anemic; no increase in opsonizing power was detected until 24 or 48 hours after the inoculation, at which time an increase was evident.

A third group is represented by Rabbits E^1 and F^1. Here repeated sublethal inoculations were made. The first injections were followed by results similar to those in Rabbits C^1 and D^1; namely, an initial increase in agglutinins and a gradual rise in opsonins and agglutinins from the 24th to the 48th hour on. The second injections were followed by a marked increase in both opsonins and agglutinins, beginning at the 24th to the 48th hour without any intervening negative phase.

A separate group of rabbits was used for the bactericidal tests, as it was often difficult to obtain enough blood from one rabbit at the various bleedings for all the tests. Protocols representing these experiments are tabulated in Table III.

The results of Experiment 3 (Table III) show a marked increase in the bactericidal power of the sera obtained from the blood taken at short and frequent intervals after the bacilli were inoculated. Five- or tenfold increase was noted 3 hours after the injection, and the increase was still greater at the expiration of 24 hours. The control, Rabbit C^2, was used to determine whether the variations observed with the sera from the inoculated rabbits were due to the different ages of the sera at the time of the tests, for they were from 1 to 24 hours old when tested. The results obtained suggest rather that remaining on the clot over night increases the bactericidal power of the sera. At least it may be said that the age of the sera was not the cause of the effects obtained. It is also evident that the sera of different rabbits vary considerably in bactericidal as they do in opsonic and agglutinating capacity. The increase in bactericidal power is not so pronounced when fewer bacilli are inoculated; $\frac{1}{80}$ or $\frac{1}{40}$ of an agar slant caused only a slight increase. It appears that a severe intoxication is necessary to cause this mobilization of the bactericidal substances.

TABLE III.

Rabbit A².

Injection.	Time of bleeding and injection.	Serum.	No. of bacilli killed.
		cc.	
⅒ agar slant in vein.	Before injection.	0.2	100
	3 hrs. after "	0.2	10,000
	5 " " "	0.2	10,000
	24 " " "	0.2	1,000,000

Rabbit B².

Injection.	Time of bleeding and injection.	Serum.	No. of bacilli killed.
		cc.	
⅒ agar slant in vein.	Before injection.	0.2	10,000
	3 hrs. after "	0.2	50,000
	5 " " "	0.2	1,000,000
	This rabbit died 5¼ hrs. after injection.		

Rabbit C².

Injections.	Time of bleeding and injection.	Serum.	No. of bacilli killed.
		cc.	
No injection.	Blood taken when the other rabbits were bled and tested at the same time.		
	Before injection.	0.2	1,000,000
	3 hrs. after "	0.2	1,000,000
	5 " " "	0.2	100,000
	24 " " "	0.2	100,000

DISCUSSION.

The data presented in this paper show conclusively that inoculation of typhoid bacilli causes no reduction in the concentrations of the natural or acquired antibodies present within the blood. Notwithstanding the fact that a decrease was expected, it was found that neither subcutaneous nor intraperitoneal nor intravenous injections sufficed to bring about the negative phase of Wright. It might have been supposed that large intravenous injections would remove the anti-

bodies from the blood as large quantities of cultures do from the serum *in vitro;* but no such effect was detected. This result may receive partial explanation from the fact that bacteria are quickly removed from the circulation through agglutination and accumulation in the organs and tissues,[6] but obviously the quantity of agglutinin thus engaged affects but little the whole quantity present.

Attention is especially drawn to the fact not only of the absence of the negative phase, but to the actual increase of the natural antibodies following the intravenous inoculations of the bacilli. The time required—from 1 to 3 hours—for the increase to become manifest is obviously too short for actual production to occur; the only alternative seems, therefore, a mobilization of preformed antibodies from the internal organs. Just what the source of the mobilized antibodies is has not been determined. The inoculations cause extensive destruction of leukocytes; but whether their disintegration liberates the antibodies cannot be stated. It is convenient to imagine that the beneficial effects said to follow the treatment of typhoid fever by means of the intravenous injection of specially prepared typhoid bacilli may be ascribed to this rapid mobilization of antibodies.

SUMMARY.

The subcutaneous, intraperitoneal, or intravenous inoculation of cultures of typhoid bacilli did not cause, as far as could be determined, a decrease in the antibody content of the blood serum of the rabbit.

On the other hand, the intravenous inoculation of typhoid bacilli causes a rapid mobilization of normal antibodies, thus increasing their concentration in the blood, to be followed somewhat later, as in the other forms of inoculation, by the production of so called acquired antibodies.

No such condition as the negative phase of Wright was discovered, although especially looked for in the experiments.

I am indebted to Miss Ida W. Pritchett for technical assistance in connection with the above study.

[6] Bull, C. G., *Jour. Exper. Med.*, 1915, xxii, 475.